



Little neighbours in Hamburg: free-living aquatic flatworms (Platyhelminthes)

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Abstract

Free-living flatworms, also known as turbellarians, are abundant and key components of aquatic habitats. They play vital roles in food webs and contribute to ecosystem health. However, our understanding of their diversity remains limited, even from comparably well-studied regions of Europe. In this work, we summarise the available records on aquatic turbellarians from Hamburg, Germany, provide new records based on material obtained during exploratory collecting, and place selected species in a molecular phylogenetic context. We sampled four localities in Hamburg, two urban and two suburban, and three other localities in Germany and Switzerland, collecting submerged vegetation, litter, and mosses. Prior to our investigation, Hamburg had documented 26 species of aquatic turbellarians. Our collections have led to the first recorded instances of *Stenostomum gotlandense* and *Castrella alba* in Germany, along with four new species for Hamburg: *Macrostomum rostratum*, *Microdalyellia schmidtii*, *Krumbachia hiemalis*, and *Polycelis tenuis*. Additionally, we provide updated information for five species previously recorded from Hamburg: *S. leucops*, *Prorhynchus stagnalis*, *Microdalyellia armigera*, *Gyratrix hermaphroditus*, and *Planaria torva*. The phylogenetic analysis revealed cryptic diversity within *S. grande* and *S. leucops*, with *S. grande* comprising two distinct clades (Brazil and Japan + Germany), and *S. leucops* consisting of four clades (Sweden + Germany, two from Finland, and Brazil). One of the Finnish clades, *S. leucops aquariorum*, is both morphologically and molecularly distinct, and we recognise it as a valid species, now *S. aquariorum*. Future research is needed to further clarify the relationships of the remaining clades of these two species, particularly with material from their type localities in the United States. Among rhabdocoel microturbellarians, we identified cryptic diversity within *Castrella truncata* (with European and North American lineages) and *Microdalyellia armigera* (encompassing Finnish and Spanish lineages). We also provided the first molecular evidence supporting the monophyly of *Krumbachia* following the sequencing of *K. hiemalis*. Conversely, species of *Bothromesostoma* were found to be nested within a clade containing species of *Mesostoma*, leading us to propose synonymising these genera. Overall, our study underscores the rich biodiversity potential of urban and suburban ecosystems for detecting freshwater turbellarian species, while highlighting the need for further research in Hamburg and across Germany to clarify species distributions. Moreover, molecular phylogenetic analyses have uncovered cryptic diversity in several species of catenulids and rhabdocoels, emphasizing the importance of future efforts to describe potential new species.

Key Words

Catenulida, cryptic diversity, Macrostomorpha, Prorhynchida, Proseriata, Rhabdocoela, Tricladida, turbellarian, urban biodiversity

Introduction

Free-living flatworms (Platyhelminthes) represent a fascinating yet understudied component of freshwater and terrestrial ecosystems worldwide. Their diverse morphologies, behaviours, and ecological roles render them invaluable subjects for understanding fundamental principles of biodiversity and ecosystem functioning (Schockaert et al. 2008; Kolasa and Tyler 2010; Dumont et al. 2014). Despite their ecological importance, the knowledge of free-living flatworm diversity remains fragmented and incomplete (see Diez et al. 2023). Historically overshadowed by their parasitic counterparts (Neodermata), free-living flatworms have often been overlooked in biodiversity surveys and taxonomic studies. However, recent advancements in molecular techniques, coupled with increased emphasis on freshwater and terrestrial ecosystem conservation, have sparked a renewed interest in elucidating the distribution, ecology, and evolutionary relationships of these enigmatic organisms (Benítez-Álvarez et al. 2023a; Brand 2023). Furthermore, there is also a much increased research effort in recent years, which can largely be attributed to the reported invasions of freshwater and land planarians (e.g. Benítez-Álvarez et al. 2023b; Justine et al. 2024).

In Germany, with its strong focus on biodiversity documentation and conservation efforts, the exploration of free-living flatworm diversity holds particular significance. The free-living flatworm fauna of Germany is, by far, the best studied worldwide since several classical taxonomists working on free-living flatworms were based in these regions. More than 400 species have been recorded from this country (Tyler et al. 2006–2024). Particularly, the marine fauna of Sylt, in the North Sea, is impressively well documented and includes more than 246 species (Armonies 2018, 2020, 2023). However, some regions of Germany have been poorly studied regarding free-living flatworm diversity, such as Hamburg. Very few studies documented and described specimens in this German region (Düren and Ax 1993; Müller and Faubel 1993) and others offered species lists (e.g. Volk 1903; Caspers 1953; Riemann 1965, 1966), recording 26 species. Most of these species are limnic but some of those from the Elbe river can be considered oligohaline due to the marine influence in the river. Hamburg is a relevant area for freshwater conservation due to its large number of protected areas (Behörde für Umwelt, Klima, Energie und Agrarwirtschaft – Abteilung Naturschutz Hamburg, 2024), which are impacted by human activities such as the development of the largest German harbour in the Elbe river. Hamburg offers a variety of freshwater habitats, including tidal flats, rivers, streams, lakes, ponds, harbour basins, irrigation ditches and others.

Germany's diverse array of habitats, ranging from pristine mountain streams to urbanized landscapes (Potschin and Bastian 2004; Bruns and Stemmer 2018), provides a unique opportunity to investigate the ecological preferences and adaptive strategies of free-living flatworms across

varied environmental gradients. Furthermore, the country's long-standing tradition of citizen science initiatives and collaborative research networks (Richter et al. 2018; Liu et al. 2021) offers a robust framework for engaging both amateur enthusiasts and professional scientists in the documentation and monitoring of flatworm diversity.

In this paper, we aim to synthesize existing knowledge on the free-living, aquatic flatworms from Hamburg, Germany. We record for the first time two species new to Germany and four species new to Hamburg, and provide new information for five species previously recorded to Hamburg. Finally, we reconstructed the molecular phylogenetic relationships for selected species of Catenulida and Rhabdocoela.

Material and methods

Sampling

The current research started with the revision of the available literature documenting the free-living, aquatic flatworms from Hamburg (see Table 1). Later, we conducted exploratory samplings in both urban and suburban ecosystems of the city. Samplings were conducted in the suburban forest of Wittenberg, Rissen (53°33'53"N, 09°45'04"E) (April 2, 2023), exploring the habitats formed by the accumulation of water on tree's holes. Secondly, submerged vegetation of Wandse river (53°34'28.8"N, 10°03'14.6"E) (September 6, 2023) and litter from the park Planten un Blomen (53°33'31.9"N, 09°59'04.6"E) (March 4, 2024), within Hamburg City were collected. Finally, a suburban farming area was sampled; samples were taken from a shallow irrigation ditch on a moist meadow in Kirchwerder-Fünfhausen (53°27'19.2"N, 10°08'28.9"E) (March 19, 2024).

Additional samplings, in order to collect relevant material for molecular analyses, were conducted in Switzerland: Röserental, Basel (47°29'35.0"N, 07°41'37.8"E) (April 12, 2024), and other German localities: Groß Glienicker lake, Brandenburg (52°28'25.7"N, 13°06'57.6"E) (March 26, 2024); and near List, Sylt (55°02'03.1"N, 08°25'07.8"E) (June 24, 2024).

The flatworms were extracted by oxygen depletion (Schockaert 1996). Afterwards, they were studied alive and specimens with hard genital structures were whole mounted with lactophenol. Specimens of species without sclerotised structures and some of the ones with hard structures were preserved in absolute ethanol for molecular analyses. Drawings of the hard parts were made with a camera lucida on a Leica DM 2500 microscope, using Nomarski interference contrast. Measurements were taken along the central axis of the measured object. For the classification of the sclerotised atrial structures in species of Polycystididae, we followed Artois and Schockaert (2003). Voucher material is stored in the Museum of Nature – Zoology, Hamburg (ZMH), which is part of the Leibniz Institute for the Analysis of Biodiversity Change (LIB).

Table 1. Checklist of the freshwater turbellarians from Hamburg, Germany, including new records.

Group	Species	Localities	Habitat	References
Catenulida	<i>Rhynchoscolex simplex</i> Leidy, 1852	Elbe river, Hamburg Harbor (Köhlbrand)	medium sand with little detritus	Riemann 1966
		Elbe river, Bank near Hamburg	detritus-free medium sand	Riemann 1966
			-	Riemann 1966
	<i>Stenostomum ciliatum</i> Kepner & Carter, 1931	Elbe river	littoral	Volk 1903
	<i>Stenostomum gotlandense</i> Larsson & Willems, 2010	Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
Macrostomorpha	<i>Stenostomum grabbskogense</i> Luther, 1960	Elbe river, Krauel	muddy substrates covered with algae	Müller and Faubel 1993
	<i>Stenostomum leucops</i> (Duges, 1828) Schmidt, 1848	Elbe river	littoral	Volk 1903
		Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
	<i>Microstomum lineare</i> (Müller, 1773) Schmidt, 1848	Elbe river, Krauel	muddy substrates covered with algae	Müller and Faubel 1993
		Elbe river	littoral	Volk 1903
Tricladida	<i>Macrostomum rostratum</i> Papi, 1959	Wandse river	vegetation with organic matter, 0.1 m deep	This study
	<i>Macrostomum hystrix</i> Ørsted, 1843	Elbe river	littoral	Volk 1903
	<i>Dendrocoelum lacteum</i> (Müller, 1774) Ørsted, 1844	Außen- and Binnenalster	-	Caspers 1953
		Elbe river	littoral	Volk 1903
	<i>Planaria torva</i> (Müller, 1773) Müller, 1776	Außen- and Binnenalster	littoral	Volk 1903
Proseriata		Elbe river	littoral	Volk 1903
		Wandse river	vegetation with organic matter, 0.1 m deep	This study
	<i>Polycelis nigra</i> (Müller, 1774) Ehrenberg, 1831	Planten un Blumen park	rotten vegetation, 0.2 m deep	This study
		Elbe river	littoral	Volk 1903
	<i>Polycelis tenuis</i> Ijima, 1884	Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
	<i>Boreusyrtis neiswestnovae</i> (Riemann, 1965) Likhnev, Koroleva, Kirilchik & Timoshkin, 2017	Elbe river, near Hamburg-Harburg	muddy substrate, 2–8 m deep	Riemann 1965
	<i>Coelogygnopora schulzii</i> Meixner, 1938	Elbe river, near Hamburg-Harburg	sandy beach, intertidal	Düren and Ax 1993
	<i>Paramonotus hamatus</i> (Jensen, 1878) Meixner, 1938	Elbe river, near Hamburg-Harburg	sandy beach, intertidal	Düren and Ax 1993
		Hamburg Harbour	intertidal, detritus-free medium sand	Riemann 1965
			subtidal, muddy substrate	Riemann 1965
Prolecithophora	<i>Pseudosyrtis subterranea</i> (Ax, 1951) Ax, 1956	Elbe river, near Hamburg-Harburg	sandy beach, intertidal	Düren and Ax 1993
	<i>Plagiostomum lemani</i> Forel & Du Plessis, 1874	Elbe river	littoral	Volk 1903
Prorhynchida	<i>Prorhynchus stagnalis</i> Schultze, 1851	Elbe river	littoral	Volk 1903
Rhabdocoela		Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
	<i>Baicalellia brevituba</i> (Luther, 1918) Nasonov, 1930	Elbe river, Krauel	muddy substrates covered with algae	Müller and Faubel 1993
	<i>Castrella alba</i> Luther, 1955	Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
	<i>Microdalyellia armigera</i> (Schmidt, 1862) Gieysztor, 1938	Elbe river	littoral	Volk 1903
		Wandse river	vegetation with organic matter, 0.1 m deep	This study
	<i>Microdalyellia schmidtii</i> (Graff, 1882) Gieysztor, 1938	Wittenberg, Rissen	tree holes filled with water, 20–50 cm over the ground level	This study
	<i>Phaenocora gracilis</i> (Vejdovsky, 1895) Graff, 1909	Elbe river	littoral	Volk 1903
	<i>Phaenocora typhlops</i> (Vejdovsky, 1880) Hofsten, 1907	Elbe river, Krauel	muddy substrates covered with algae	Müller and Faubel 1993
	<i>Phaenocora unipunctata</i> (Ørsted, 1843) Bendl, 1908	Elbe river	littoral	Volk 1903
	<i>Olisthanella truncula</i> (Schmidt, 1858) Voigt, 1892	Elbe river	littoral	Volk 1903
	<i>Mesostoma ehrenbergii</i> (Focke, 1836) Ørsted, 1843	Elbe river	littoral	Volk 1903

Group	Species	Localities	Habitat	References
	<i>Mesostoma lingua</i> (Abildgaard, 1789) Schmidt, 1848	Elbe river	littoral	Volk 1903
	<i>Mesostoma tetragonum</i> (Müller, 1774) Schmidt, 1848	Elbe river	littoral	Volk 1903
	<i>Krumbachia hiemalis</i> Schwank, 1979	Wittenberg, Rissen	tree holes filled with water, 20–50 cm over the ground level	This study
	<i>Gyratrix hermaphroditus</i> Ehrenberg, 1830	Elbe river, near Hamburg-Harburg	sandy beach, intertidal	Ax 1957
		Elbe river	littoral	Volk 1903
		Wandse river	vegetation with organic matter, 0.1 m deep	This study
		Kirchwerder-Fünfhausen	submerged vegetation and litter	This study
	<i>Placorhynchus dimorphus</i> Karling, 1947	Elbe river Krauel	muddy substrates covered with algae	Müller and Faubel 1993

DNA extraction, amplification and sequencing

Total DNA of selected taxa was extracted using a protocol for low-input tissues (Laumer 2023). The specimens were processed with 195 µl of the TNES lysis buffer and 5 µl of proteinase K (Invitrogen) at 55 °C for 1 hour. Subsequently, 1.5 µl yeast tRNA (Invitrogen) as coprecipitant, 65 µl 5M NaCl, and 290 µl 96% EtOH were added to the lysed. Then the samples were stored at -20 °C for 2 hours. Later, the lysed was centrifuged (18 000 rpm) and the supernatant was discarded. Purification was performed with two 70% EtOH wash steps and, finally, the DNA was resuspended overnight at 4 °C in 0.1X TE buffer with 0.02% Tween™ 20 (Thermofisher).

For the selected specimens (Catenulida and Rhabdocoela), we amplified partial sequences of the 18S and 28S ribosomal DNA genes (rDNA). Additionally, for catenulids a fragment of the COI mitochondrial DNA gene (mDNA) was also amplified. Amplification primers and protocols varied according to the taxa (Catenulida or Rhabdocoela) (Table 2). The PCR products were verified on a 1% agarose gel, stained with

HD Green Plus (Intas Science Imaging), and purified with ExoCleanUp FAST (Avantor). Sequencing was carried out by Macrogen Europe B.V. (Amsterdam) under BigDye™ terminator cycling conditions on an ABI3730XL DNA Sequencer.

Molecular phylogenetic analyses

Obtained contigs were quality-trimmed (error probability = 0.05) and assembled in Geneious Prime v2025.0 (Kearse et al. 2012). Consensus sequences were subjected to a BLAST search (Altschul et al. 1990) on the NCBI website (<https://ncbi.nlm.nih.gov>) to check for signs of contamination. To detect potential pseudogenes, newly obtained COI mDNA sequences were translated in Geneious (translation Table 5) and visually screened for the presence of stop codons. Available sequences relevant for our phylogenetic analyses were mined from GenBank (see Table 3 for Catenulida and Table 4 for Rhabdocoela) (Benson et al. 2012). Separate datasets were compiled for catenulids: 18S (43 sequences) and 28S rDNA (35 sequences), and COI mDNA (47 sequences),

Table 2. Primer sequences and protocols used for PCR amplification.

Primer	Direction	Primer sequence (5'-3')	Reference
Rhabdocoela subunit 18S			
TimA	Forward	AMCTG GTT GAT CCT GCCAG	Willems et al. (2006)
TimB	Reverse	TGATCCATCTGCAGGTTACCT	Willems et al. (2006)
95 °C * 5 min 10 s, 30X (94 °C * 30 s, 55 °C * 30 s, 72 °C * 90 s), 72 °C * 5 min			
Rhabdocoela subunit 28S			
LSU5	Forward	TAG GTC GAC CCG CTG AAY TTA	Van Steenkiste et al. (2013)
LSU6-3B	Reverse	GAG AAG GGT TCC ATG TGA ACAGC	Van Steenkiste et al. (2013)
95 °C * 5 min 10 s, 30X (94 °C * 60 s, 50 °C * 60 s, 72 °C * 90 s), 72 °C * 5 min			
Catenulida subunit 18S			
U95947-0025	Forward	CATATGCTTGTCTCAAAG	Yamasaki et al. 2012
U95947-1788	Reverse	GGAAACCTTGTTACGACT	Yamasaki et al. 2012
94 °C * 3 min, 35X (94 °C * 1 min, 50 °C * 1 min, 72 °C * 2 min), 72 °C * 8 min			
Catenulida subunit 28S			
LSU5	Forward	TAG GTC GAC CCG CTG AAY TTA	Larsson and Jondelius 2008
L1642R	Reverse	CCAGCGCCATCCATTTTCA	Larsson and Jondelius 2008
94 °C * 3 min, 38X (94 °C * 30 s, 50 °C * 30 s, 72 °C * 30 s), 72 °C * 5 min			
Catenulida subunit COI			
COI5B	Forward	TTCTGRTTYTTYGGNCAY CC	Rosa et al. 2015
COI3B	Reverse	AAGTGTGNGGRARAANGT	Rosa et al. 2015
95 °C * 5 min, 30X (94 °C * 1 min, 50 °C * 1 min, 72 °C * 1 min), 72 °C * 3 min			

Table 3. GenBank accession numbers of Catenulida used in the study (*new sequences).

Species	18S rDNA	28S rDNA	COI	Distribution
<i>Stenostomum arevaloi</i> K04_53	FJ384808	FJ384847	FJ384889	Sweden
<i>S. arevaloi</i> K04_80	FJ384810	FJ384849	FJ384895	Sweden
<i>S. arevaloi</i> K05_60	FJ384833	FJ384871	FJ384910	Sweden
<i>S. bryophilum</i> K04_09	FJ384799	FJ384836	FJ384875	Sweden
<i>S. bryophilum</i> K04_30	FJ196320	FJ384843	FJ384882	Sweden
<i>S. bryophilum</i> K04_49	–	FJ384845	FJ384888	Sweden
<i>S. bryophilum</i> K04_71	FJ196333	FJ196343	FJ384892	Sweden
<i>S. bryophilum</i> K04_78	FJ196334	–	FJ384894	Sweden
<i>S. gotlandense</i> K04_81	FJ384811	FJ384850	FJ384896	Sweden
<i>S. gotlandense</i> K04_90	–	FJ384855	FJ384900	Sweden
<i>S. gotlandense</i> K04_93	–	FJ384856	FJ384901	Sweden
<i>S. gotlandense</i> K04_94	–	–	FJ384902	Sweden
<i>S. gotlandense</i> D-259	*PQ722290	*PQ722307	*PQ687017	Hamburg, Germany
<i>S. grabbskogense</i> K04_06	FJ196326	FJ196337	FJ384874	Sweden
<i>S. grabbskogense</i> K04_11	FJ196327	FJ196338	FJ384876	Sweden
<i>S. grabbskogense</i> K04_12	–	FJ384838	FJ384877	Sweden
<i>S. grabbskogense</i> K04_15	–	FJ384839	FJ384878	Sweden
<i>S. grabbskogense</i> K04_19	FJ384801	FJ384841	FJ384880	Sweden
<i>S. grabbskogense</i> K05_27	FJ384827	FJ384867	FJ384907	Sweden
<i>S. grande</i>	–	–	KM056359	Brazil
<i>S. grande</i>	AB665104	–	AB665116	Japan
<i>Stenostomum</i> sp. G-11	*PQ722293	*PQ722310	–	Brandenburg, Germany
<i>Stenostomum</i> sp. G-12	*PQ722294	*PQ722311	–	Brandenburg, Germany
<i>Stenostomum</i> sp. S-260	*PQ722296	*PQ722313	–	Sylt, Germany
<i>S. handoelense</i> K05_17	FJ384823	FJ384864	FJ384905	Sweden
<i>S. handoelense</i> K05_20	FJ384824	FJ384865	FJ384906	Sweden
<i>S. heebuktense</i> K04_45A	FJ196330	–	FJ384887	Sweden
<i>S. leucops</i>	–	–	KJ476143	Brazil
<i>S. leucops</i> AJ405976	–	–	AJ405976	Finland
<i>S. leucops</i> D-229	*PQ722295	*PQ722312	–	Sylt, Germany
<i>S. leucops</i> D-257	*PQ722291	*PQ722308	–	Hamburg, Germany
<i>S. leucops</i> D-258	*PQ722292	*PQ722309	–	Hamburg, Germany
<i>S. leucops</i> K04_18	FJ384800	FJ384840	FJ384879	Sweden
<i>S. leucops</i> K04_29	FJ384804	FJ384842	FJ384881	Sweden
<i>S. leucops</i> K04_63A	–	–	FJ384891	Sweden
<i>S. leucops</i> K04_75	FJ384809	FJ384848	FJ384893	Sweden
<i>S. leucops</i> K04_85	FJ384812	FJ384852	FJ384898	Sweden
<i>S. leucops</i> K04_87	FJ384813	FJ384853	FJ384899	Sweden
<i>S. leucops</i> K05_51	FJ384830	FJ384869	FJ384908	Sweden
<i>S. leucops</i> K05_55	FJ384832	FJ384870	FJ384909	Sweden
<i>S. leucops aquariorum</i> AJ012519	AJ012519	–	–	Finland
<i>S. saliens</i> 1	AB665114	–	AB665123	Japan
<i>S. saliens</i> 2	AB665115	–	AB665124	Japan
<i>S. simplex</i> 1	AB665105	–	AB665117	Japan
<i>S. simplex</i> 2	AB665107	–	AB665118	Japan
<i>S. simplex</i> 3	AB665109	–	AB665119	Japan
<i>S. simplex</i> 4	AB665110	–	AB665120	Japan
<i>S. simplex</i> S-227	–	–	*PQ687018	Sylt, Germany
<i>S. sphagnetorum</i> K04_01	FJ384797	–	FJ384873	Sweden
<i>S. sphagnetorum</i> K05_07	FJ384818	FJ384860	FJ384904	Sweden
<i>S. steveoi</i> K04_59	FJ196331	–	FJ384890	Sweden
<i>S. steveoi</i> K04_84	–	FJ384851	FJ384897	Sweden
<i>S. tuberculosum</i> 1	–	–	AB665121	Japan
<i>S. tuberculosum</i> 2	–	–	AB665122	Japan
<i>S. tuberculosum</i> 3	AB665112	–	–	Japan
<i>S. tuberculosum</i> 4	AB665113	–	–	Japan
<i>Catenula turgida</i> K04_32	FJ384805	FJ196339	FJ384883	Sweden
<i>Rhynchoscolex simplex</i> K04_41	FJ384806	FJ384844	FJ384885	Sweden

and rhabdocoels: 18S (98 sequences) and 28S rDNA (72 sequences). Following the results of Larsson et al. (2008), *Catenula turgida* (Zacharias, 1902) Larsson, Ahmadzadeh & Jondelius, 2008 and *Rhynchoscolex simplex* Leidy, 1852 were included as outgroup for the analyses of *Stenostomum*. Based on previous studies (Van Steenkiste et al. 2021; Diez et al. 2023), we selected two species of Temnocephalidae: *Halammovortex* sp. and

Table 4. GenBank accession numbers of Rhabdocoela used in the study (*new sequences).

Species	18S rDNA	28S rDNA
<i>Acrochordonoposthia conica</i>	KC529487	KC529617
<i>Bothromesostoma personatum</i>	KC529501	–
<i>Bothromesostoma personatum</i>	M58347	–
<i>Bothromesostoma</i> sp.	D85098	–
<i>Bryoplana xerophila</i>	KC529489	KC529619
<i>Carcharodopharynx</i> sp.	KC529481	KC529612
<i>Castrada hofmanni</i>	KC529496	–
<i>Castrada intermedia</i>	KC529497	–
<i>Castrada lanceola</i>	AY775751	–
<i>Castrada luteola</i>	AY775752	–
<i>Castrada neocomensis</i>	KC529498	–
<i>Castrada viridis</i>	AY775753	–
<i>Castrella alba</i> D-282	*PQ722297	*PQ722314
<i>Castrella alba</i> D-283	*PQ722298	*PQ722315
<i>Castrella alba</i> G-29	*PQ722299	PQ722316
<i>Castrella pinguis</i>	KC529438	KC529569
<i>Castrella truncata</i>	AY775777	KC529570
<i>Castrella truncata</i> Switzerland B-21	*PQ722300	*PQ722317
<i>Dalyellia tatrlica</i>	KC529443	KC529574
<i>Dalyellia viridis</i>	KC529444	KC529575
Dalyelliidae n. gen. n. sp.	KC529441	–
<i>Dochmiotrema limicola</i>	KC529495	KC529624
<i>Dochmiotrema</i> sp.	PP723168	PP723167
<i>Gieysztoria acaraiia</i>	KC529470	KC529601
<i>Gieysztoria ashokae</i>	KC529466	KC529597
<i>Gieysztoria beltrani</i>	KC529475	KC529606
<i>Gieysztoria</i> cf. <i>billabongensis</i>	KC529442	KC529573
<i>Gieysztoria</i> cf. <i>cuspidata</i>	KC529457	KC529588
<i>Gieysztoria choctaw</i>	KC529476	KC529607
<i>Gieysztoria complicata</i>	KC529473	KC529604
<i>Gieysztoria cuspidata</i>	KC529458	KC529589
<i>Gieysztoria dodgei</i>	KC529479	KC529610
<i>Gieysztoria garudae</i>	KC529467	KC529598
<i>Gieysztoria iberica</i>	KC529461	KC529592
<i>Gieysztoria infundibuliformis</i>	KC529468	KC529599
<i>Gieysztoria knipovici</i>	KC529463	–
<i>Gieysztoria ornata</i>	KC529460	KC529591
<i>Gieysztoria pavimentata</i>	KC529472	KC529603
<i>Gieysztoria rubra</i>	KC529480	KC529611
<i>Gieysztoria</i> sp. n. 1 scissors	KC529454	KC529585
<i>Gieysztoria</i> sp. n. 2 spine	KC529455	KC529586
<i>Gieysztoria</i> sp. n. 3 aberrant	KC529456	KC529587
<i>Gieysztoria</i> sp. n. 4 indian	KC529464	KC529595
<i>Gieysztoria</i> sp. n. 5 red	KC529469	KC529600
<i>Gieysztoria</i> sp. n. 6 ‘brown’	KC529474	KC529605
<i>Gieysztoria</i> sp. n. 7 hooklet	KC529477	KC529608
<i>Gieysztoria</i> sp. n. 8 sardinia	KC529462	KC529593
<i>Gieysztoria</i> sp. par YG 2018	MG820105.2	MG820107.2
<i>Gieysztoria</i> sp. pel YG 2018	MG820112	MG820115
<i>Gieysztoria</i> sp. ZCX 2012_1	JQ999991	–
<i>Gieysztoria</i> sp. ZCX 2012_2	JQ999992	–
<i>Gieysztoria</i> sp. ZXY 2011	HQ993097	–
<i>Gieysztoria triquetra</i>	KC529478	KC529609
<i>Gieysztoria zuluensis</i>	KC529465	KC529596
<i>Krumbachia</i> sp.	KC529488	KC529618
<i>Krumbachia hiemalis</i> D-159	*PQ722301	*PQ722322
<i>Krumbachia hiemalis</i> D-160	*PQ722302	*PQ722323
<i>Mesocastrada</i> sp.	U70081	–
<i>Mesostoma lingua</i>	AY775759	KC529626
<i>Mesostoma lingua</i> AY775759	AY775759	–
<i>Mesostoma lingua</i> KC529626	–	KC529626
<i>Mesostoma lingua</i> AJ243682	AJ243682	–

Species	18S rDNA	28S rDNA
<i>Mesostoma thamagae</i>	AY775760	–
<i>Microdalyellia armigera</i> Finland	KC529451	KC529582
<i>Microdalyellia armigera</i> Spain	KC529452	KC529583
<i>Microdalyellia brevispina</i>	KC529450	KC529581
<i>Microdalyellia fairchildi</i>	KC529447	KC529578
<i>Microdalyellia fusca</i>	KC529453	KC529584
<i>Microdalyellia kupehwieseri</i> Switzerland B-11	*PQ722303	*PQ722320
<i>Microdalyellia kupehwieseri</i> Switzerland B-12	*PQ722304	*PQ722321
<i>Microdalyellia nanella</i>	KC529449	KC529580
<i>Microdalyellia picta</i>	KC529446	KC529577
<i>Microdalyellia rossi</i>	KC529448	KC529579
<i>Microdalyellia schmidtii</i> Belgium	KC529445	KC529576
<i>Microdalyellia schmidtii</i> Hamburg D-156	*PQ722305	*PQ722318
<i>Microdalyellia schmidtii</i> Hamburg D-158	*PQ722306	*PQ722319
<i>Microdalyellia sinensis</i>	JF429837	–
<i>Microdalyellia</i> sp. ZXY 2011	HQ993095	–
<i>Olisthanella truncula</i>	KC529494	KC529623
<i>Olisthanella truncula</i> AY775761	AY775761	–
<i>Opisthomum arsenii</i>	KC529491	KC529620
<i>Phaenocora foliacea</i>	KC529492	KC529621
<i>Phaenocora shenda</i> PHE1-18	ON843455	ON843453
<i>Phaenocora shenda</i> PHE2-18	ON843456	ON843454
<i>Phaenocora</i> sp. n.	KC529493	KC529622
<i>Phaenocora unipunctata</i>	AY775762	–
<i>Protoplanella simplex</i>	KC529490	–
<i>Pseudodalyellia alabamensis</i>	KC529440	KC529571
<i>Rhynchomesostoma rostratum</i>	KC529499	KC529625
<i>Rhynchomesostoma rostratum</i> UH77.15	KC529500	–
<i>Strongylostoma devleeschouweri</i>	KC529486	–
<i>Strongylostoma elongatum</i>	AY775771	–
<i>Strongylostoma elongatum spinosum</i>	KC869830	KC869883
<i>Strongylostoma radiatum</i>	KC529485	KC529616
<i>Typhloplana viridata</i>	KC529484	KC529615
<i>Grappleria corona</i>	MW052803	MW052802
<i>Halammovortex</i> sp.	KC529437	KC529567
<i>Temnocephala fasciata</i>	KC869834	KC869888
<i>Temnosewellia minor</i>	AY157183	AY157164

Temnosewellia fasciata (Haswell, 1887) Damborenea & Cannon, 2001, and two of Jensiidae: *T. minor* (Haswell, 1887) Damborenea & Cannon, 2001 and *Grappleria corona* Van Steenkiste, Rivlin, Kahn, Wakeman & Leander, 2021, as outgroups for the analysis of the rhabdocoel families ‘Typhloplanidae’ and Dalyelliidae.

Ribosomal datasets were aligned using MAFFT v7, as implemented in Geneious (Kato and Standley 2013; Kato et al. 2017). COI dataset was translationally aligned using the MUSCLE v3.8.425 (Edgar 2004) executable implemented in Geneious. The resulting alignments were concatenated in Geneious. An initial partitioning scheme defining gene boundaries was manually constructed. Separate partitions were specified for the three codon positions in the COI alignment. The concatenated alignment and partition files were used as input for the ModelFinder tool (Kalyaanamoorthy et al. 2017) on the IQ-TREE webserver (Trifinopoulos et al. 2016). Model fit was evaluated using the Akaike Selection Criterion and partition merging was enabled (Lanfear et al. 2012). The latter feature determines the best-fit partitioning scheme for a

particular dataset, while also calculating the best-fitting evolutionary models for each selected subset.

Maximum likelihood (ML) analyses were conducted using the ‘Tree Inference’ tool on the IQ-TREE server (Nguyen et al. 2015), using edge-linked partitions. Branch support was assessed by ultrafast bootstrapping (UFboot) (Hoang et al. 2017) and the nonparametric approximate likelihood-ratio test (SH-aLRT) (Guindon et al. 2010), both with 1000 replicates. Bayesian inference (BI) was carried out using the Metropolis-coupled Markov Chain Monte Carlo (MC³) algorithm, implemented in MrBayes v3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway (Miller et al. 2010). Best-fitting partitions and substitution models were specified where possible; otherwise, the second most complex model implemented in MrBayes was chosen. Two independent runs were conducted simultaneously for 10,000,000 generations, each including one cold and three heated chains. Trees were sampled every 1000th generation, the first 25% being discarded as burn-in. Chain convergence was confirmed by the average standard deviation of split frequencies dropping below 0.01, the potential scale reduction factor approaching 1.0, and the log probability reaching a stationary distribution. Obtained topologies were summarised in a majority-rule consensus tree. Inferred posterior probabilities (pp) were employed as support values. ML and BI trees were visualised and rooted in FigTree v1.4.4 (Rambaut 2006–2019). Weakly supported clades (pp < 0.95, SH-aLRT < 80, and UFboot < 95) were collapsed.

Table 5. Best-fitting partitions and substitution models used in the phylogenetic analyses, as calculated in ModelFinder (Kalyaanamoorthy et al. 2017) according to AICc.

Dataset	Partition scheme	Substitution model	MrBayes friendly model
Rhabdocoela	18S, 28S rDNA	GTR+F+I+G4	GTR+F+I+G4
Catenulida	18S rDNA	TIME+G4	SYM+G4
	28S rDNA	TIM+F+G4	GTR+F+G4
	1 st and 2 nd codon positions of COI	GTR+F+I+G4	GTR+F+I+G4
	3 rd codon positions of COI	TIM+F+G4	GTR+F+I+G4

Results

Aquatic turbellarians from Hamburg

The literature review revealed the presence of 26 species of aquatic turbellarians previously documented in Hamburg (Table 1). Among these, the most diverse taxon is Rhabdocoela, comprising 11 species. Additionally, between one and four species have been documented in the following taxa: Catenulida, Macrostomorpha, Tricladida, Proseriata, Prorhynchida, and Prolecithophora. The species records span the time between 1903 and 1993. The majority of these findings originate from the Elbe river, with only a few specimens collected in smaller rivers and

tributaries of the Elbe. The localities sampled during our survey differ from those previously studied.

Based on our collected material, the catenulid *Stenostomum gotlandense* Larsson & Willems, 2010 and the rhabdocoel *Castrella alba* Luther, 1955 are recorded for the first time from Germany. Four species are recorded for the first time from Hamburg: *Macrostomum rostratum* Papi, 1959, *Microdalyellia schmidtii* (Graff, 1882) Gieysztor, 1938, *Krumbachia hiemalis* Schwank, 1979, and *Polycelis tenuis* Ijima, 1884. Five known species, previously recorded to Hamburg were also collected during our field work: *Stenostomum leucops* (Duges, 1828) Schmidt, 1848, *Prorhynchus stagnalis* Schultze, 1851, *Gyratrix hermaphroditus* Ehrenberg, 1831, *Microdalyellia armigera* (Schmidt, 1862) Gieysztor, 1938, and *Planaria torva* (Müller, 1773). Considering our findings, the current number of aquatic turbellarians known from Hamburg is 31 species.

Taxonomy

Platyhelminthes Minot, 1876
Catenulida Meixner, 1924
Stenostomidae Vejdvsky, 1880
***Stenostomum* Schmidt, 1848**

***Stenostomum gotlandense* Larsson & Willems, 2010**

Fig. 1

Known distribution. Species only known, until now, from Gotland, Sweden (Larsson et al. 2008; Larsson and Willems 2010).

Material. Two specimens studied alive and stored in absolute ethanol for molecular analyses, one of them sequenced; collected in Kirchwerder-Fünfhausen, submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep.

Remarks. Specimens measuring 768–957 µm long ($\bar{x}$ = 863 µm; n = 2) and 90–110 µm at widest point ($\bar{x}$ = 100 µm; n = 2), with two zooids, slender, tapering to both rounded extremes (Fig. 1A). The ciliated pits (Fig. 1B: cp) are relative short and open close to the most anterior part of the body. The epidermis is fully ciliated. Larger cilia are distributed along the body, particularly in the anterior and posterior ends. The brain (Fig. 1B: br) consists of two pairs of lobes, the anterior brain (Fig. 1C: ab) and the posterior brain (Fig. 1C: pb). We were not able to determine the exact number of compartments of the anterior brain, but in one specimen there appear to exist seven. Refractile bodies not present. Proximal rim of the pharynx (Fig. 1A, B: ph) with a number of folds and surrounds the large mouth opening (Fig. 1A, B: m). The protonephridium (Fig. 1D: pn) ends in a nephridiopore at the posterior end of the body.

As noted by Larsson and Willems (2010), the folded rim of the pharynx, the large mouth opening, and the small ciliated pits represent a unique combination of morphological traits within *Stenostomum*. Our morphological identifica-

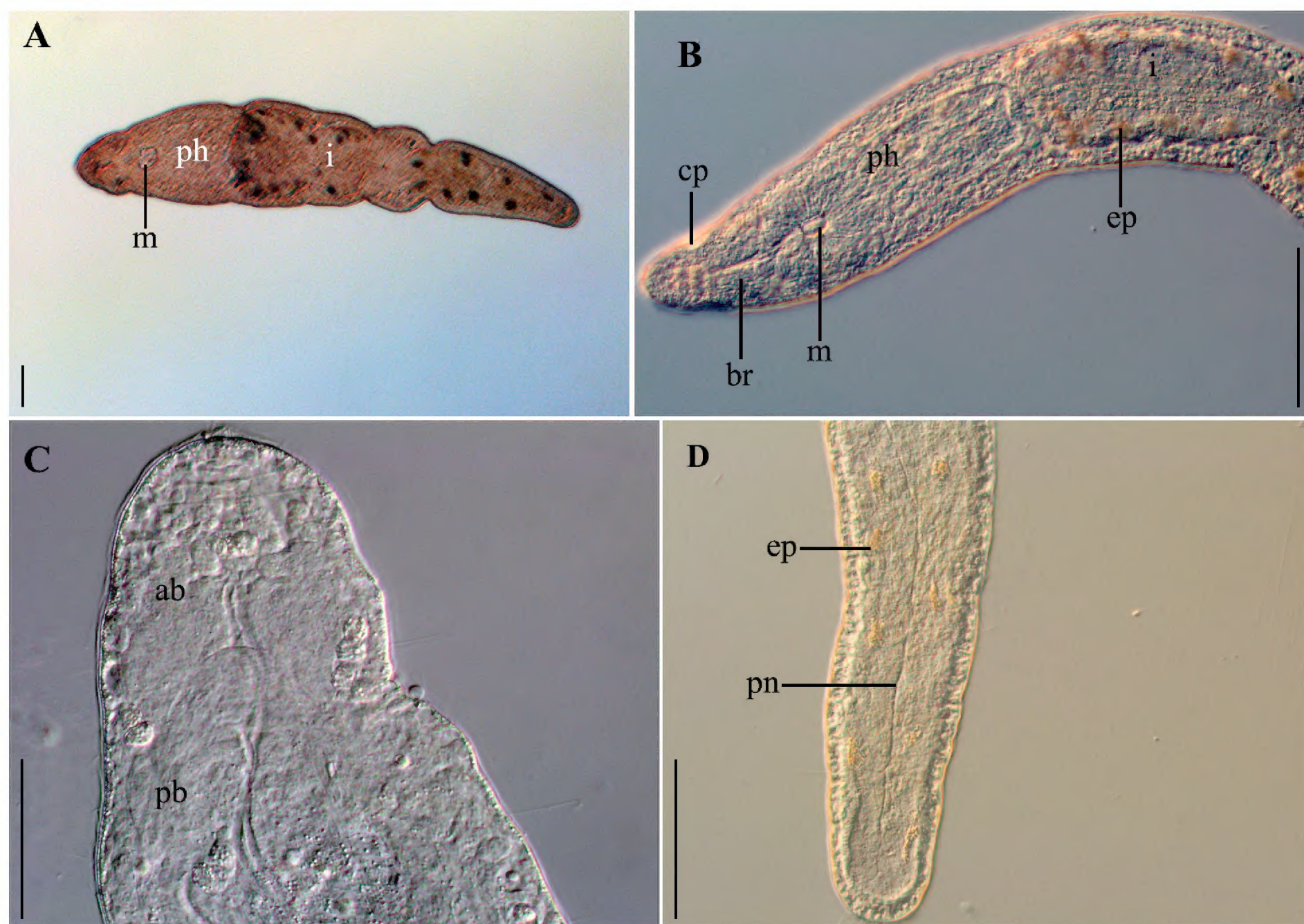


Figure 1. *Stenostomum gotlandense*. **A.** Habitus of a swimming specimen; **B.** Anterior part of the body; **C.** Anterior part of the body showing the brain; **D.** Posterior part of the body. Abbreviations: **ab** anterior brain; **br** brain; **cp** ciliated pits; **ep** excretophore; **i** intestine; **m** mouth; **pb** posterior brain; **ph** pharynx; **pn** protonephridia. Scale bars: 50 µm (**A**, **C**); 100 µm (**B**, **D**).

tion is further corroborated by the phylogenetic analysis, leading us to confidently report this species for the first time in Germany, specifically in Hamburg. The German specimens are similar in size, considering the length of the first zooid (495–588 µm), to those from Sweden (500 µm). However, Larsson and Willems (2010) observed that their specimens exhibited approximately 10 compartments in the anterior brain, whereas the specimens from Hamburg present about seven. Nonetheless, one specimen illustrated by Larsson and Willems (2010: fig. 3B) shows only six segments in the anterior brain.

Stenostomum leucops (Duges, 1828) Schmidt, 1848

Fig. 2

Known distribution. Species with a broad distribution through North America (United States and Mexico) (Higley 1918; Nuttycombe and Waters 1938; Kolasa et al. 1987; Núñez-Ortiz et al. 2016; Glasgow 2021), South America (Argentina, Brazil, Peru, and Suriname) (Marcus 1945; van der Land 1970; Noreña-Janssen 1995; Gamo and Leal-Zanchet 2004; Noreña et al. 2005; Damborenea et al. 2011; Reyes et al. 2021), Iceland and Faroe Islands (Steinböck 1948), West Europe (United Kingdom, Ireland, The Netherlands, Germany, Switzerland,

and Spain) (Graff 1882; Hofsten 1911; Gieysztor 1931; Young 1970, 1972, 1973; Noreña et al. 2007), East Europe (Poland, Romania, Serbia, and Croatia) (Graff 1882; Steinböck 1933; Kolasa 1971; Mack-Fira 1974), Russia (Nasonov 1926; Steinböck 1932; Timoshkin et al. 2010), Asia (Thailand and Japan) (Yamazaki et al. 2012; Ngamniyom and Panyarachun 2016), and Africa (Tanzania and Kenya) (Young and Kolasa 1974; Young 1976).

Material. Three specimens studied alive and stored in absolute ethanol; collected in Kirchwerder-Fünfhausen, submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep. Three specimens studied alive and stored in absolute ethanol; collected in Groß Glienicker lake, littoral, floating vegetation. Three specimens studied alive and stored in absolute ethanol; collected in Sylt, floating vegetation in a small pond. Two specimens from Kirchwerder-Fünfhausen and one from Sylt were sequenced for the molecular analyses.

Remarks. Specimens measuring 1120–1490 µm long ($\bar{x}$ = 1305 µm; n = 2) and 175–220 µm at widest point ($\bar{x}$ = 198 µm; n = 2), with two zooids, anterior end rounded and posterior tapering (Fig. 2A–C). The ciliated pits (Fig. 2D: cp) are relative short and open close to the most anterior part of the body. The epidermis is fully ciliated. Larger cilia are distributed along the body, particularly in the anterior and posterior ends. The brain (Fig. 2A, B, D: br) consists of

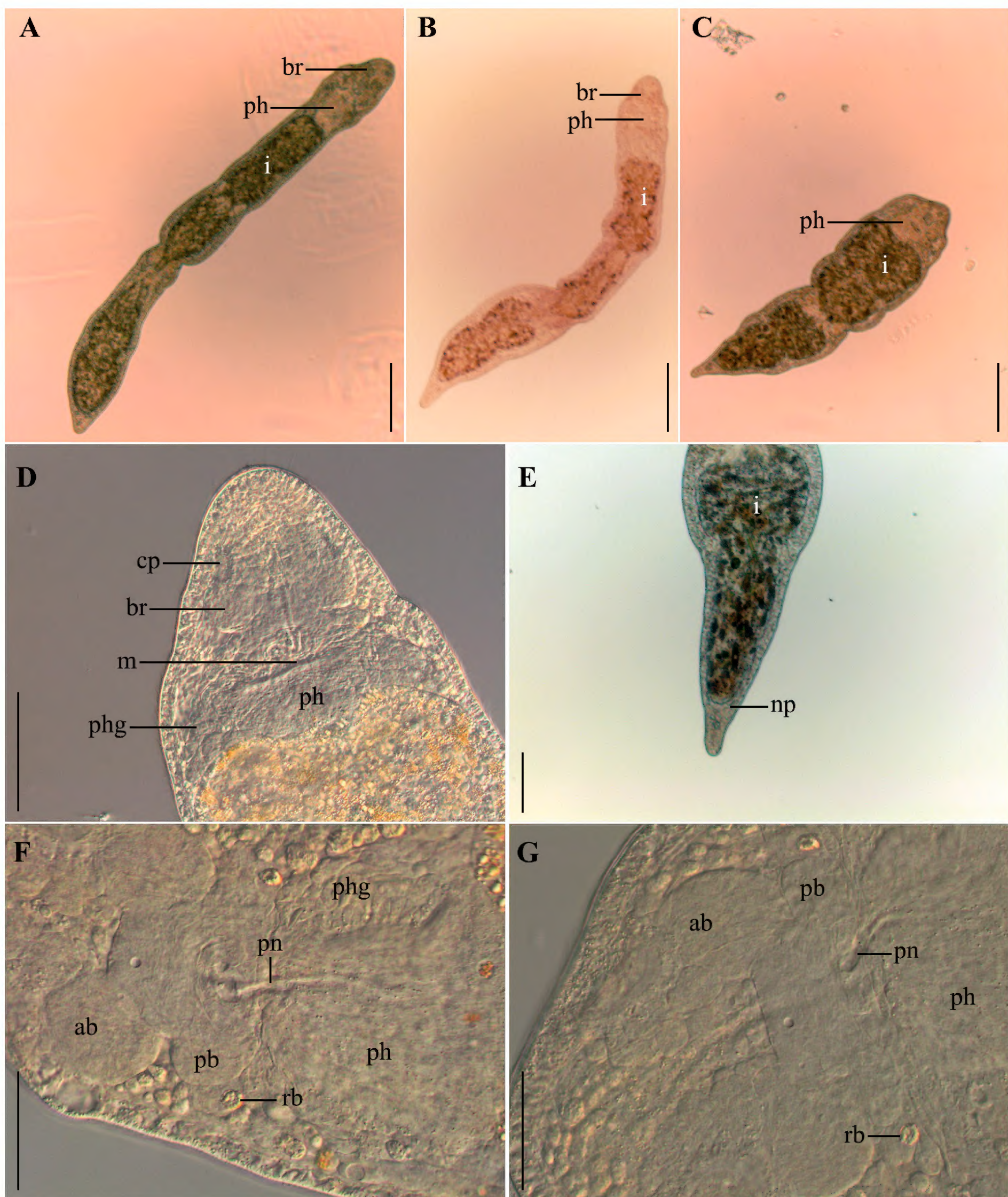


Figure 2. *Stenostomum leucops*. **A–C** Habitus of swimming specimens; **D**. Anterior part of the body; **E**. Posterior part of the body; **F, G**. Anterior part of the body. Abbreviations: **ab** anterior brain; **br** brain; **cp** ciliated pits; **i** intestine; **m** mouth; **np** nephridiopore; **pb** posterior brain; **ph** pharynx; **phg** pharyngeal glands. Scale bars: 200 µm (**A–C**); 100 µm (**D, E**); 50 µm (**F, G**).

two pairs of lobes, the anterior brain (Fig. 2F, G: ab) and the posterior brain (Fig. 2F, G: pb). The anterior brain is more distinct but does not show a clearly compartments. A pair of refractile bodies (Fig. 2F, G: rb) are connected to the posterior brain via stalked structures. The refractile bodies are 8–9 µm in diameter ($n = 3$). The number of spherules contained in the refractile bodies is difficult to observe but

they are more than 20 in all studied specimens. The refractile bodies can appear either rounded or crescent-shaped, depending on the orientation of these structures.

Our identification of specimens was primarily based on Luther's (1960) description. However, recent studies have suggested that *S. leucops* may actually represent a complex of cryptic species, possibly related to *S. grande*

Child, 1902 (see Yamazaki et al. 2012; Rosa et al. 2015). Molecular evidence supports this notion and echoes earlier discussions by Nuttycombe and Waters (1938) and Marcus (1945), who both deemed the available description insufficient for clear recognition of the species. Rosa et al. (2015) identified three distinct clades within *S. leucops*, each distributed in Brazil, the United Kingdom, and Sweden, respectively, but were unable to find morphological diagnostic traits to differentiate them. The phylogenetic analysis here developed (see section Molecular phylogenetic analyses) shows a close relationship between *S. leucops* from Germany and Sweden.

Given that the type locality of *S. leucops* is in the vicinity of New York, United States, a comprehensive morphological and molecular phylogenetic analysis of specimens from that area is essential to stabilize the classification of *S. leucops* (see Discussion).

Rhabditophora Ehlers, 1985

Macrostomorpha Doe, 1986

Macrostomidae Beneden, 1870

Macrostomum Schmidt, 1848

***Macrostomum rostratum* Papi, 1951**

Fig. 3

Known distribution. Species with a broad known distribution including Europe (United Kingdom, The Netherlands, Germany, Finland, Spain, and Italy) (Papi 1951; Luther 1960; Rixen 1961; Young 1970, 1973; Tulp 1974; Farias et al. 1996; Noreña et al. 2007), Russia (Luther 1960), and Kenya (Young 1976).

Material. Two specimens studied alive, one preserved in ethanol for future molecular analyses, the second cut in two pieces, the posterior part containing the stylet for whole mounting and the anterior part preserved for molecular analyses. Collected in Wandse river, submerged vegetation with organic matter, 0.1 m deep.

Description. Animals 0.8–0.9 mm long ($n = 2$), unpigmented and with a pair of eyes (Fig. 3A, B: e). General morphology corresponds to that in previously described specimens (see Papi 1951). Epidermis fully ciliated, with some larger cilia distributed though the body. False seminal vesicle absent and true seminal vesicle (Fig. 3C–E: sv) opening into the prostate vesicle (vesicula granulorum). The prostate vesicle (Fig. 3C–F: pv) opens proximally into the stylet (Fig. 1A, C–F: st). One specimen had a fully developed stylet, measuring 60 μm in length; it is hook shaped and with the distal part twisted; aperture subdistal and dorsal. Well-developed eggs observed (Fig. 3A, C: eg).

According to the drawings of Papi (1951: figs 32–33) the stylet of this species measures 42–65 μm , which is in the range of our collected specimen. In other populations recorded from Germany, the stylet is 65–77 μm long (Rixen 1961). The most characteristic traits for this species are the distal turn and the dorsal aperture of the stylet; therefore, we identify our specimen as *M. rostratum*.

Prorhynchida Karling, 1974

Prorhynchidae Hallez, 1894

***Prorhynchus* Schultze, 1851**

***Prorhynchus stagnalis* Schultze, 1851**

Fig. 4

Known distribution. Species with a worldwide distribution, recorded from South America (Brazil) (Marcus 1944; Reyes et al. 2021), North America (United States) (Higley 1918; Kenk 1949; Kolasa et al. 1987; Smith 1991; Glasgow 2021), Faroes Islands (Steinböck 1931), West Europe (United Kingdom, Ireland, Sweden, Belgium, Netherlands; Germany, Switzerland, Austria, Poland, Czech Republic, France, Spain) (Schultze 1851; Graff 1882, 1913; Fuhrmann 1894, 1900; Steinböck 1923; Southern 1936; Rixen 1961; Young 1970, 1972, 1973; Kolasa 1971; Farias et al. 1996; Noreña et al. 2007), East Europe (Bulgaria, Hungary, Hungary, North Macedonia-Albania, Russia) (Graff 1913; Steinböck 1932; An der Lan 1939), Asia (China and Japan) (Okugawa 1953; Peng et al. 2007), and Africa (Kenya) (Young and Young 1976).

Material. Six specimens studied alive, two preserved in ethanol for future molecular analyses, four cut in two pieces, the anterior part containing the stylet for whole mounting (ZMH V13839–13842) and the posterior part preserved for molecular analyses; collected in Kirchwerder-Fünfhausen, submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep.

Remarks. Animals 2.5–3.5 mm long depending on the contraction stage, opaque, without eyes (Fig. 4A). The oral pore opens at the anterior tip; the pharynx (Fig. 4B, C: ph) is located behind the brain (Fig. 4B: br) and beside the prostate vesicle (Fig. 4B, C: pv), and proximally opens into the intestine (Fig. 4A: i). The intestine of some specimens contained numerous chaetae of oligochaetes; a feeding behaviour previously recorded to the species (Tyler et al. 2018).

The ovary (Fig. 4A: ov) is very long and runs medially, extending over the posterior two thirds of the body; eggs were observed. The male reproductive system includes the large unpaired testis, located at mid body; the seminal vesicle, located posterior to the pharynx; the prostate vesicle running beside the pharynx; and the armed copulatory organ. The prostate vesicle shows thick muscle walls, perforated by the necks of the extracapsular prostatic glands, containing a coarse-granular secretion. Proximally, the prostate vesicle looks empty, maybe because it contains a very fine secretion. A very large duct connects the prostate vesicle with the armed bulb.

The armed male copulatory organ (Fig. 4D–F) is located in the anterior part of the body, anterior to the brain. It consists of a central stylet (Fig. 4B, D–F: st) surrounded by two concentric rings of spines. The stylet is 64–68 μm long ($\bar{x} = 66 \mu\text{m}$; $n = 4$), proximally funnel shaped and posteriorly tapering to the distal sharp tip. As described by Tyler et al. (2018), the stylet consists of two pieces, clearly distinguishable in the posterior third of the stylet. In the distal area, only the sharp tip of the stylet

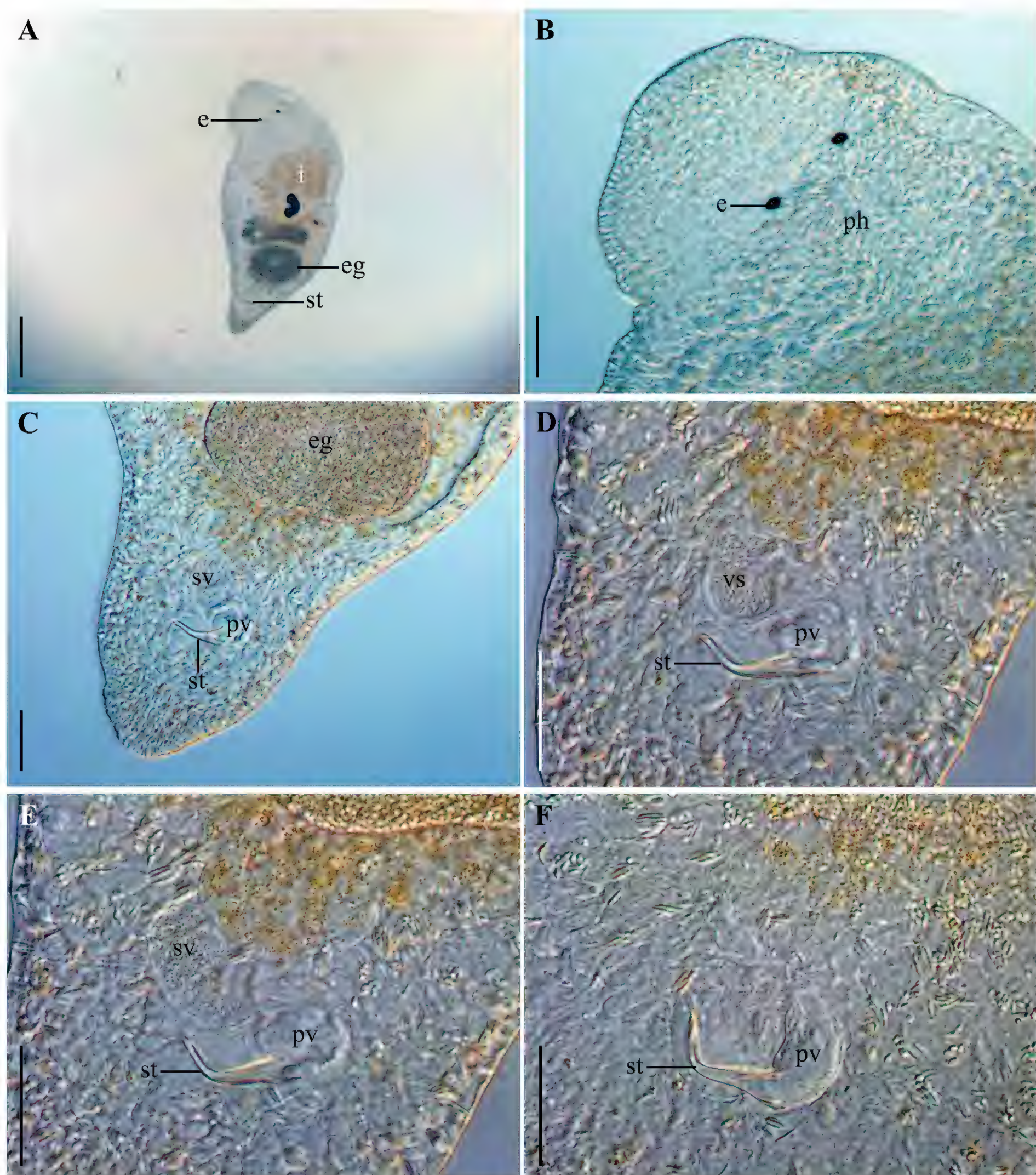


Figure 3. *Macrostomum rostratum*. **A.** Habitus of a swimming specimen; **B.** Anterior part of the body; **C–F.** Posterior part of the body. Abbreviations: **e** eye; **eg** egg; **ph** pharynx; **pv** prostate vesicle; **st** stylet; **sv** seminal vesicle. Scale bars: 200 µm (**A**); 50 µm (**B–F**).

(part of the inner tube) could be observed. The number of bars on each ring surrounding the stylet is difficult to observe; however, in two specimens it seems that there are 10 bars in the external and six in the internal ring. This pattern has also been identified in populations from the United States and Brazil (Tyler et al. 2018; Reyes et al. 2021). The external bars (Fig. 4D–F: eb) are arched, 53–57 µm long ($\bar{x} = 55$ µm; $n = 4$), proximally broad and posteriorly taper to rounded tips. The internal bars (Fig. 4D, F: ib) appear straight to slightly arched but the rest

of their morphology is hardly distinguishable; they are 47–53 µm long ($\bar{x} = 50$ µm; $n = 4$).

It has been proposed that *P. stagnalis* comprises a complex of species primarily distinguished by variations in the morphology of male sclerotised structures. However, comprehensive morphological and molecular investigations are imperative to elucidate the taxonomy of this species group fully. Furthermore, extensive sampling is required, including the type locality of the species (Greifswald, Germany). The specimens we have examined

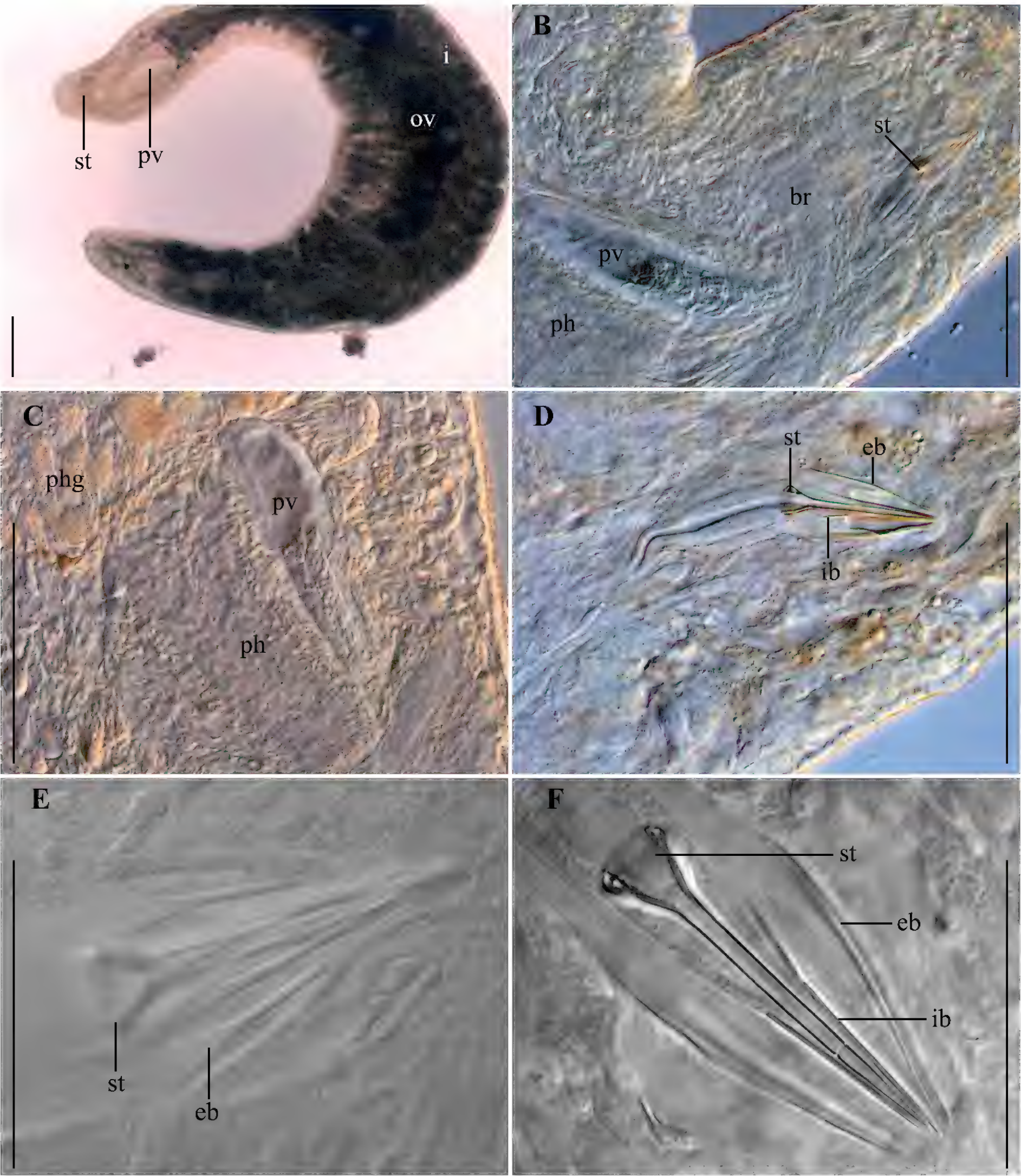


Figure 4. *Prorhynchus stagnalis*. **A.** Habitus of a swimming specimen; **B–D.** Anterior part of the body; **E, F.** Sclerotised structures. Abbreviations: **br** brain; **eb** external bars; **i** intestine; **ib** internal bars; **ov** ovary; **ph** pharynx; **phg** pharyngeal glands; **pv** prostate vesicle; **st** stylet. Scale bars: 200 μ m (**A**); 100 μ m (**B–D**); 50 μ m (**E, F**).

exhibit the smallest stylet (~66 μ m) compared to those documented in the literature from The Urals (100–124 μ m; Rogozin 2015), the United States (~90 μ m; Tyler et al. 2018), and Brazil (~123 μ m; Reyes et al. 2021).

Rhabdocoela Ehrenberg, 1831
Kalyptorhynchia Graff, 1905
Eukalyptorhynchia Meixner, 1928
Polycystididae Graff, 1905

***Gyratrix* Ehrenberg, 1831**

***Gyratrix hermaphroditus* Ehrenberg, 1831**

Fig. 5

Known distribution. This is the microturbellarian species with the widest distribution worldwide (see Artois and Tessens 2008; Diez et al. 2018). However, this distribution corresponds to a puzzle of dozens of cryptic species (Tessens et al. 2021).

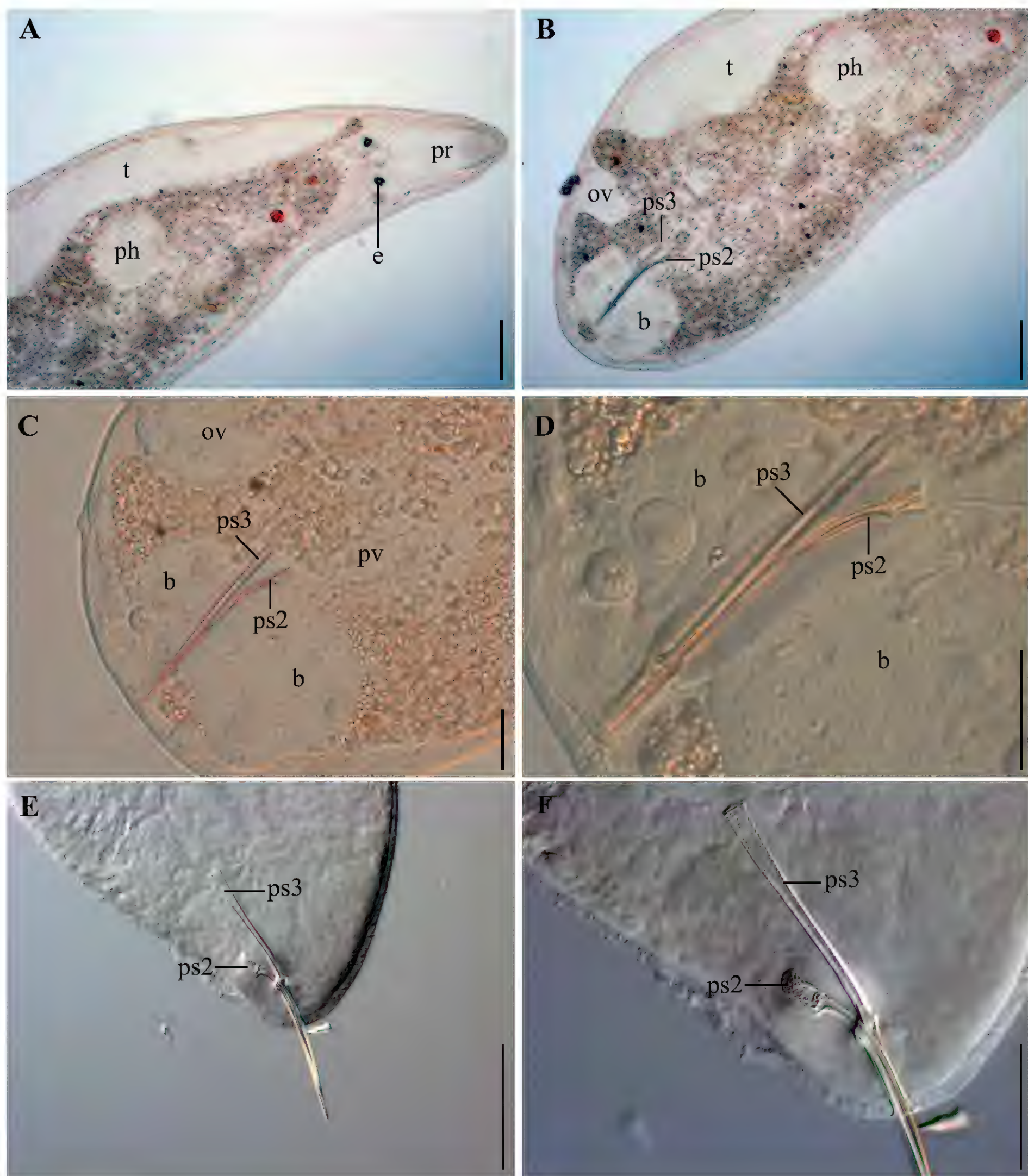


Figure 5. *Gyratrix hermaphroditus*. **A, B.** Habitus of a swimming specimen; **C–F.** Posterior part with sclerotised structures. Abbreviations: **b** bursa; **e** eye; **ov** ovary; **ph** pharynx; **pr** proboscis; ps3, ps4 prostate stylet type 3 and 4, respectively; **p**v prostate vesicle; **t** testi. Scale bars: 100 µm (**A**); 50 µm (**B–F**).

Material. Specimens were found in two locations. One specimen was studied alive and preserved in ethanol for future molecular analyses. It was collected in Wandse river, submerged vegetation with organic matter, 0.1 m deep. Fifteen specimens were studied alive, eleven of them whole mounted and four preserved for future molecular analyses collected in Kirchwerder-Fünfhausen, among submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep.

Remarks. The specimen from Wandse river is 1354 µm long and those from Kirchwerder-Fünfhausen

are 870–926 µm long ($\bar{x} = 904$ µm; $n = 6$). They are unpigmented, with a pair of eyes (Fig. 5A: e) posterior to the proboscis (Fig. 5A: pr).

The testis (Fig. 5A, B: t) is located at the left body side, extending from the region of the eyes to about three quarters of body length. Ovary and atrial organs located posteriorly in the body. The prostate stylet type II (Fig. 5B–F: ps2) is 189 µm long in the specimen from Wandse river and 155–169 µm long ($\bar{x} = 164$ µm; $n = 9$) in those from Kirchwerder-Fünfhausen. The prostate stylet type

III (Fig. 5B–F: ps3) is 147 μm long in the specimen from Wandse river and 150 μm long ($\bar{x}$ = 131–167 μm ; n = 9) in those from Kirchwerder-Fünfhausen. In the specimens from both localities, the stylet type III distally bifurcates and therefore ends in two opposed tips.

Vitellarium not observed. The ovary (Fig. 5B, C: ov) is kidney shaped in the specimen from Wandse river, with the oocytes organised in a row, increasing in diameter from proximal to distal. In contrary, in the specimens from Kirchwerder-Fünfhausen, the ovary is globular, with oocytes of dissimilar size. The bursa (Fig. 5B–D: b) is located completely posterior and overlaps with the stylets; several masses of sperm in digestion were observed.

Based on our findings, it appears that the specimens collected from the two sampled localities correspond to two distinct species within the *G. hermaphroditus* complex. These species are distinguishable by differences in the size of the sclerotised male structures and the morphology of the ovary. However, differentiation within this extensive species complex poses significant challenges and warrants molecular investigations. Phylogenetic analyses conducted by Tessens et al. (2021) revealed a remarkable diversity of 62–78 species concealed under the name *G. hermaphroditus*, with many of them confined to single localities while others are more widely distributed.

The morphology of the hard structures in our specimens appears to align with group H as defined by Tessens et al. (2021), characterised by the bifurcate ending of prostate stylet type III. Notably, this group comprises exclusively freshwater species from Australia. However, molecular studies are essential to validate this morphological resemblance. Specimens from Germany included in the aforementioned phylogenetic study fall into groups A and B (comprising freshwater and brackish species) and group L (marine species). Nonetheless, representatives from the latter three groups exhibit a prostate stylet type III ending in a simple, hook-shaped tip. Future integrative studies are imperative to unravel the intricate taxonomy of the *G. hermaphroditus* species complex, which should encompass the analysis of specimens from its type locality in Berlin, Germany.

Dalytyphloplanida Willems, Wallberg, Jondelius, Littlewood, Backeljau, Schockaert & Artois, 2006
Neotyphloplanida Willems, Wallberg, Jondelius, Littlewood, Backeljau, Schockaert & Artois, 2006
Limnotyphloplanida Van Steenkiste, Tessens, Willems, Backeljau, Jondelius & Artois, 2013
Dalyelliidae Graff, 1905
Castrella Graff, 1905

***Castrella alba* Luther, 1955**

Figs 6, 7

Known distribution. Species recorded from Finland and Sweden (Luther 1955).

Material. Seven specimens studied alive, two of which were whole mounted afterwards (ZMH V13831–13832); collected in Kirchwerder-Fünfhausen, submerged vegeta-

tion and litter in an irrigation channel, 0.1–0.2 m deep. Four specimens studied alive and preserved in absolute ethanol, collected in Groß Glienicker lake; littoral, floating vegetation. Two specimens from Kirchwerder-Fünfhausen and one from Groß Glienicker lake used for molecular phylogenetic analyses.

Remarks. Live specimens about 1 mm long, anterior margin rounded and posterior pointing, translucent, pinkish-brownish colouration due to parenchymal glands (Figs 6–7). A pair of eyes (Figs 6A–C, 7A: e) anterior to the pharynx; the eyes are formed by two pigmented spots linked by a bridge, and sometimes look like two independent structures. Barrel-shaped pharynx (Figs 6A–C, 7A: ph) 122–183 μm long ($\bar{x}$ = 147 μm ; n = 5). Testes located postero-lateral to the pharynx. The male copulatory organ includes a seminal vesicle (Fig. 7E, F: sv), a prostate vesicle (Fig. 7E, F: sv), and the stylet. The stylet (Figs 6D–F, 7C–F: st) is 43–74 μm long ($\bar{x}$ = 60 μm ; n = 3) and displays a proximal handle and a distal spiny part; a hook-shaped, well-differentiated, largest spine is 14–30 μm long ($\bar{x}$ = 22 μm ; n = 3). The vitellaria (Fig. 6B: vi) run beside the pharynx until the posterior third of the body, fusing before opening into the oviduct. The oval eggs are 64–120 μm long (n = 2) and present a 95–168 μm long stalk (n = 2) (Fig. 7A–C: eg).

As noted by Luther (1955), identifying the male sclerotised apparatus in *C. alba* is particularly challenging. In our study, we were able to distinguish a fully developed stylet in only three out of 11 specimens, while this structure was indistinguishable in the whole mounted material. The stylet in this species is generally delicate and, at first glance, appears underdeveloped. Consequently, Luther (1955) speculated that this species might be synonymous with *C. vernalis* Beklemischev, 1921. However, the newly studied material, which includes fully developed specimens, suggests that *C. alba* bears a closer resemblance to *C. truncata* (Abildgaard, 1789) Sekera, 1906, as indicated by the stylet, which has a proximal handle and a distal spiny section with one spine more developed than the others. Nevertheless, our phylogenetic analysis confirms that *C. alba* represents a distinct lineage from *C. truncata* (see section Molecular Phylogenetic Analyses; Fig. 16).

***Microdalyellia* Gieysztor, 1938**

***Microdalyellia armigera* (Schmidt, 1862) Gieysztor, 1938**
 Fig. 8

Known distribution. This species has been broadly recorded in the United States, Iceland, Faroe Islands, overall Europe (United Kingdom, Ireland, Finland, Belgium, Germany, Austria, Switzerland, Czech Republic, Poland, Romania, Bulgaria, Latvia, Italy, France, and Spain), Russia, and Japan. See a summary of this distribution and main references in Van Steenkiste et al. (2011).

Material. One specimen studied alive and whole mounted (ZMH 13833), collected in Wandse river, submerged vegetation with organic matter, 0.1 m deep.

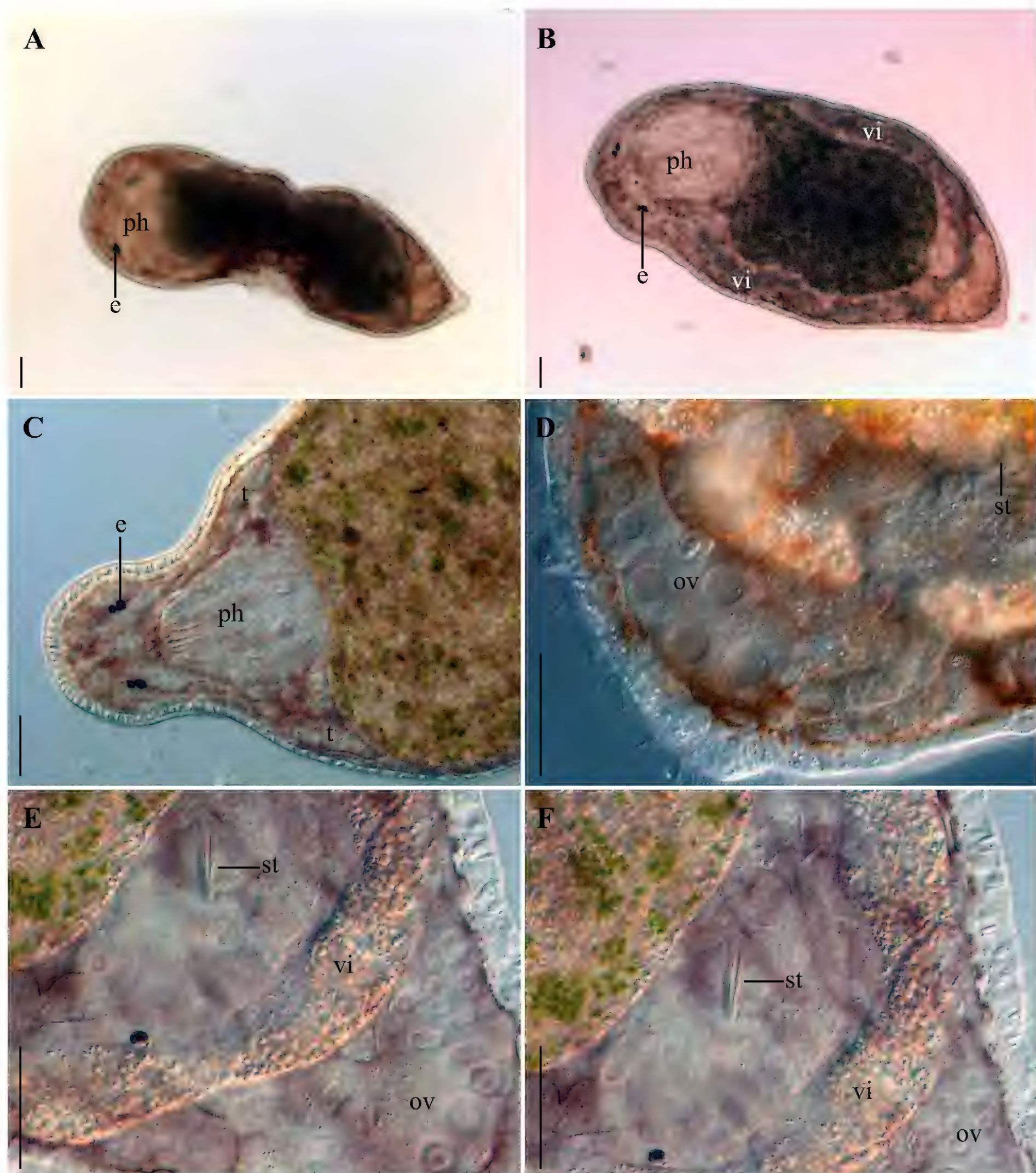


Figure 6. *Castrella alba* Luther, 1955 collected in Hamburg. **A, B.** Habitus of a swimming specimen; **C.** Details of the anterior part of the body; **D–F.** Posterior part of the body with atrial structures. **e** eye; **ov** ovary; **ph** pharynx; **st** stylet; **vi** vitellaria. Scale bars: 50 μ m.

Remarks. Live animal about 1 mm long, anterior margin rounded and posterior pointing, translucent, orange colouration due to parenchymal glands (Fig. 8A). A pair (Fig. 8A: e) of eyes anterior to the pharynx. Barrel-shaped pharynx (Fig. 8A: ph) 250 μ m long. Testes located antero-lateral to the male copulatory bulb. This bulb encompasses a seminal vesicle (Fig. 8B: sv), a prostate vesicle (Fig. 8B: sv), and the stylet. The stylet (Fig. 8C–E: st) is 123 μ m long, H-shaped as typical for species of *Microdalyellia*. One arm carries five 17- μ m-long spines, and the other a single 58- μ m-long spine. The vitellaria (Fig. 8A:

vi) run beside the pharynx until the posterior third of the body, fusing before opening into the oviduct. The oval egg is 140 μ m long (Fig. 8A, C–F: eg).

Microdalyellia armigera shows a high morphological variability of the male sclerotised stylet, the main character to differentiate species in most microturbellarians. However, it would not be surprising if this variability represents cryptic speciation, as suggested by our phylogenetic analysis (see section Molecular Phylogenetic Analyses; Fig. 16). For example, the specimens of *M. armigera* collected by Müller and Faubel (1993) in the Elbe river

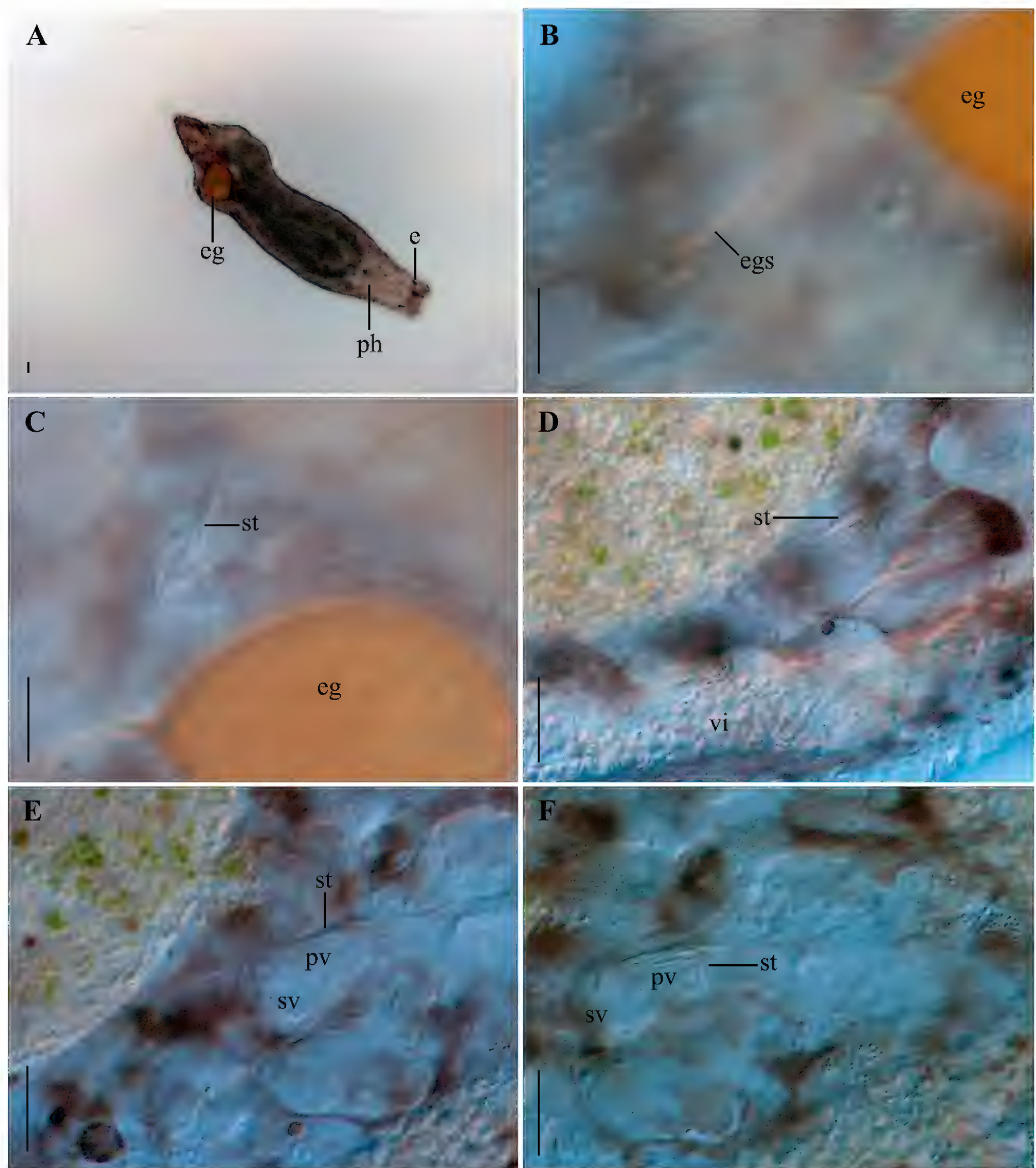


Figure 7. *Castrella alba* Luther, 1955 collected in Brandenburg. **A.** Habitus of a swimming specimen; **B.** Details of an egg and its stalk; **C.** Details of an egg and the stylet; **D–F.** Posterior part of the body with atrial structures. **e** eye; **eg** egg; **egs** egg stalk; **ph** pharynx; **st** stylet; **vi** vitellaria. Scale bars: 50 µm.

present a stylet much larger than in our specimen (220 µm vs 123 µm respectively) and have 3–9 spines in one arm. The size of this structure in other North-European populations varies between 82 µm and 130 µm (Luther 1955; Rixen 1961) and in specimens from Spain is 165 µm long (Van Steenkiste et al. 2011). In general the number of spines in both arms can be variable (see Luther 1955; Bauchhenss 1971) but commonly there is one arm with one spine and the other with 4–5 (Müller and Faubel 1993).

***Microdalyellia schmidtii* (Graff, 1882) Gieysztor, 1938**
Figs 9, 10

Known distribution. Species with a known distribution in West Europe (United Kingdom, Finland, Germany, and Switzerland) (Graff 1882, 1913; Fuhrmann 1894; Luther 1955; Rixen 1961; Young 1970), and Russia (Nasonov 1919, 1926).

Material. Observations on eight live animals, five whole mounted afterwards (ZMH 13834–13838) and the

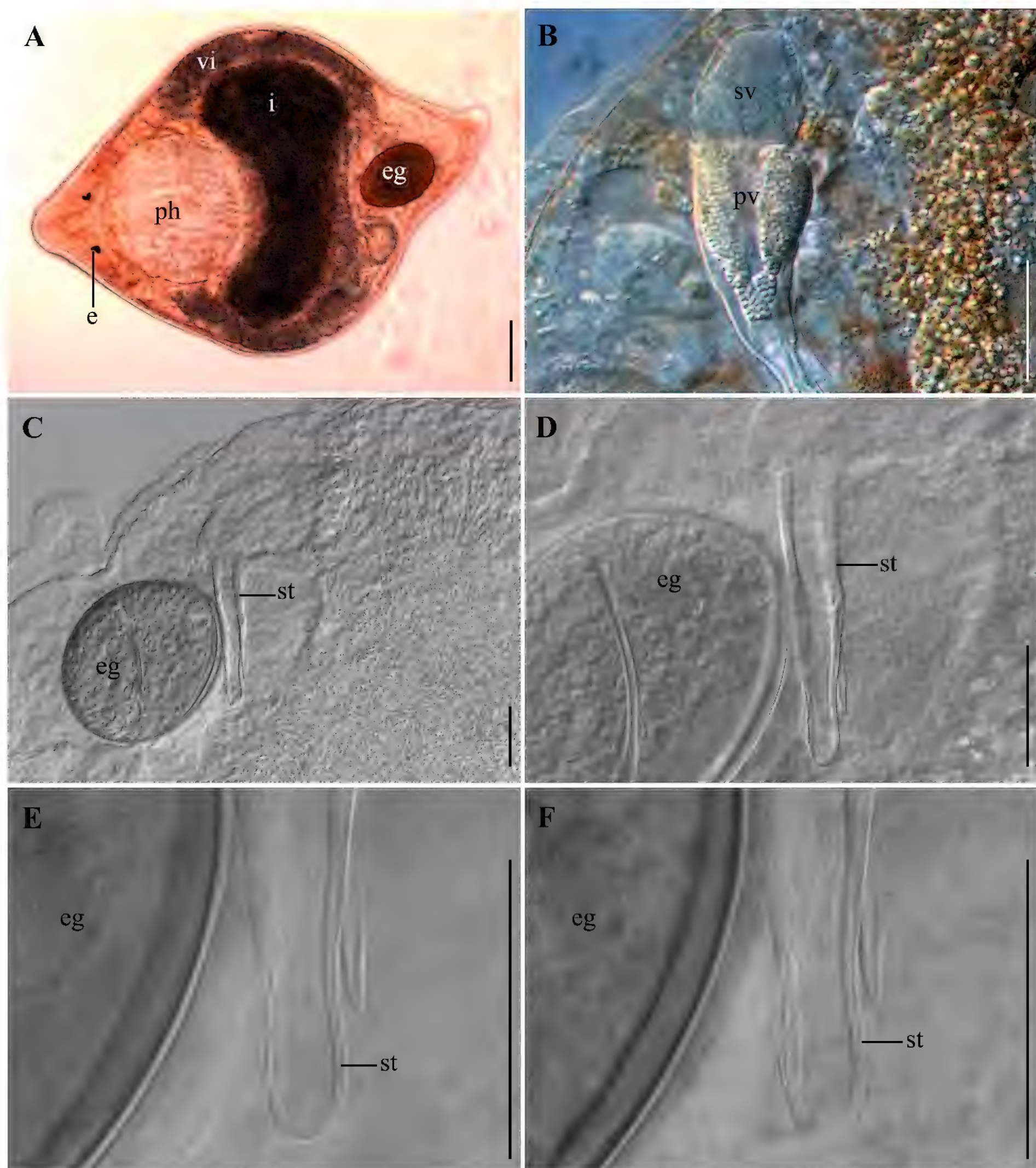


Figure 8. *Microdalyellia armigera*. **A.** Habitus of a swimming specimen; **B.** Seminal and prostate vesicles; **C–F.** Male stylet. **e** eye; **eg** egg; **i** intestine lumen; **ph** pharynx; **pv** prostate vesicle; **st** stylet; **sv** seminal vesicle; **vi** vitellaria. Scale bars: 100 µm (A); 50 µm (B–F).

other three preserved in absolute ethanol (two already sequenced for molecular analyses); collected in Wittenberg, Rissen, on tree holes filled with water and litter, 20–50 cm over the ground level.

Remarks. Animals 704–1010 µm long ($\bar{x}$ = 840 µm; n = 3), with a pair of eyes (Fig. 9A: e) anterior to the pharynx. The yellowish to orange colouration is due to parenchymal glands. The barrel-shaped pharynx (Fig. 9A: ph) is 231–289 µm long ($\bar{x}$ = 262 µm; n = 3), when the animal is not contracted.

The atrial organs are located in the posterior body third. The pair of testes (Fig. 9B, C: t) is located beside the male copulatory organ. The copulatory organ encompasses a seminal vesicle (Fig. 9B, C: sv), a prostatic vesicle (Fig. 9B, C: pv), and the stylet. The stylet (Figs 9B, C: st, E, F, 10) is 147–151 µm long ($\bar{x}$ = 149 µm; n = 4) and each arm is armed with a conical spine. The larger spine is 64–75 µm long ($\bar{x}$ = 69 µm; n = 4) and the smaller is 48–52 µm long ($\bar{x}$ = 50 µm; n = 4), both with the distal tip bent outside and hook shaped. Each

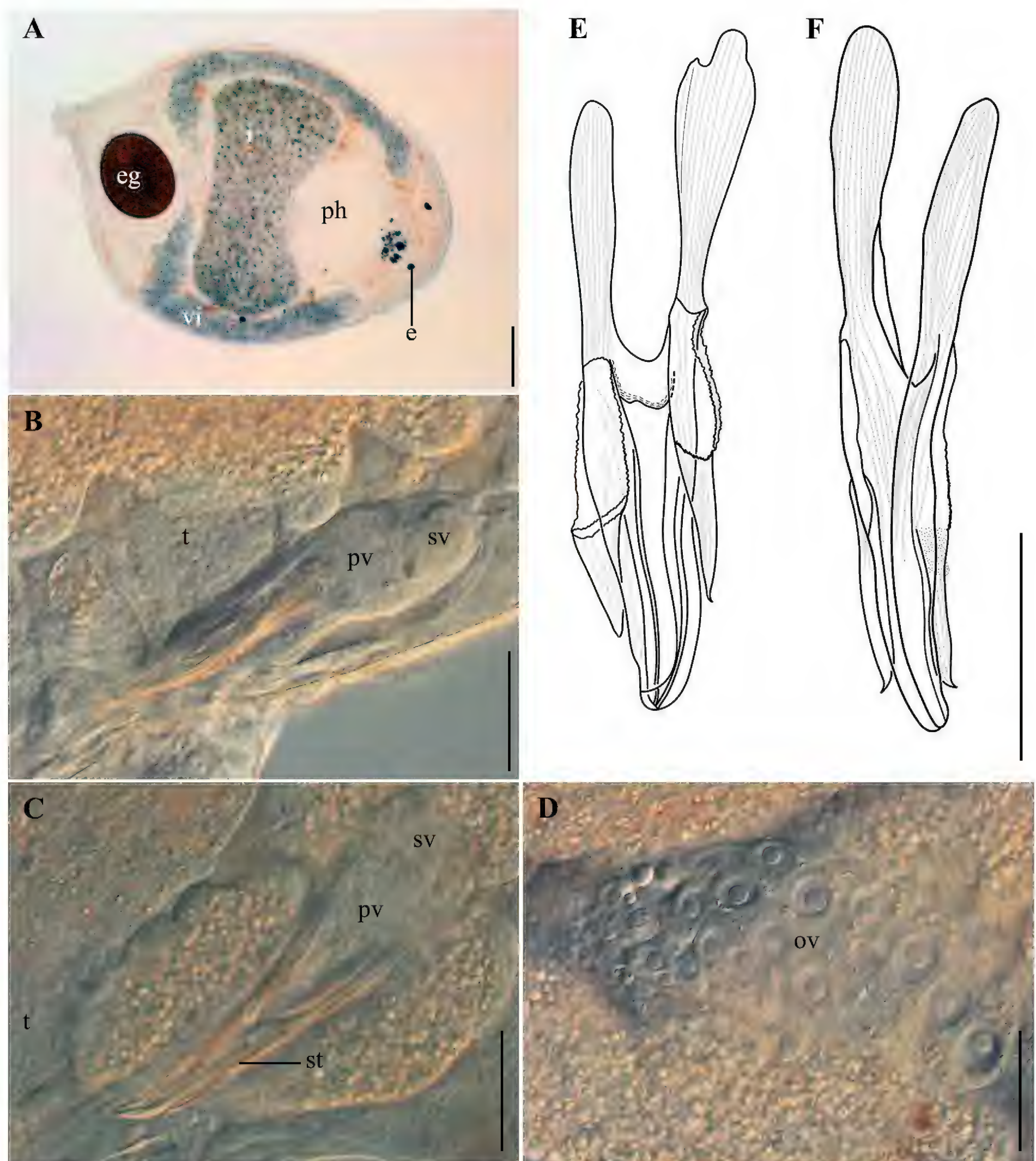


Figure 9. *Microdalyellia schmidtii*. **A.** Habitus of a swimming specimen; **B, C.** Male atrial organs; **D.** Ovary; **E, F.** Male stylet. Scale bars: 100 µm (**A**); 50 µm (**B–F**).

vitellarium (Fig. 9A: vi) runs beside the pharynx until the posterior third of the body, and fuses before entering the oviduct. The ovary (Fig. 9D: ov) is 157–212 µm long ($\bar{x}$ = 185 µm; n = 2). The distal most three oocytes are of similar size and organised in a row, the rest of the ovary is full of smaller oocytes with different degrees of development. The eggs (Fig. 9A: eg) are oval, 148–206 µm long ($\bar{x}$ = 177 µm; n = 4).

Around 44 species of *Microdalyellia* have been recorded globally (Tyler et al. 2006–2024). Among them,

M. schmidtii stands out as the only one possessing a single, funnel-shaped spine on each arm of the stylet, leading to its stylet being described as ‘plow-shaped’ (Graff 1882; Luther 1955). However, doubts have been raised regarding the validity of this species due to its similarity to *M. armigera* and *M. kupelwieseri* (Meixner 1915; Ruebush and Hayes 1939). It has been suggested that *M. schmidtii* and *M. kupelwieseri* might be forms of *M. armigera* with a greatly reduced number of spines (Luther 1955; Young 1970).

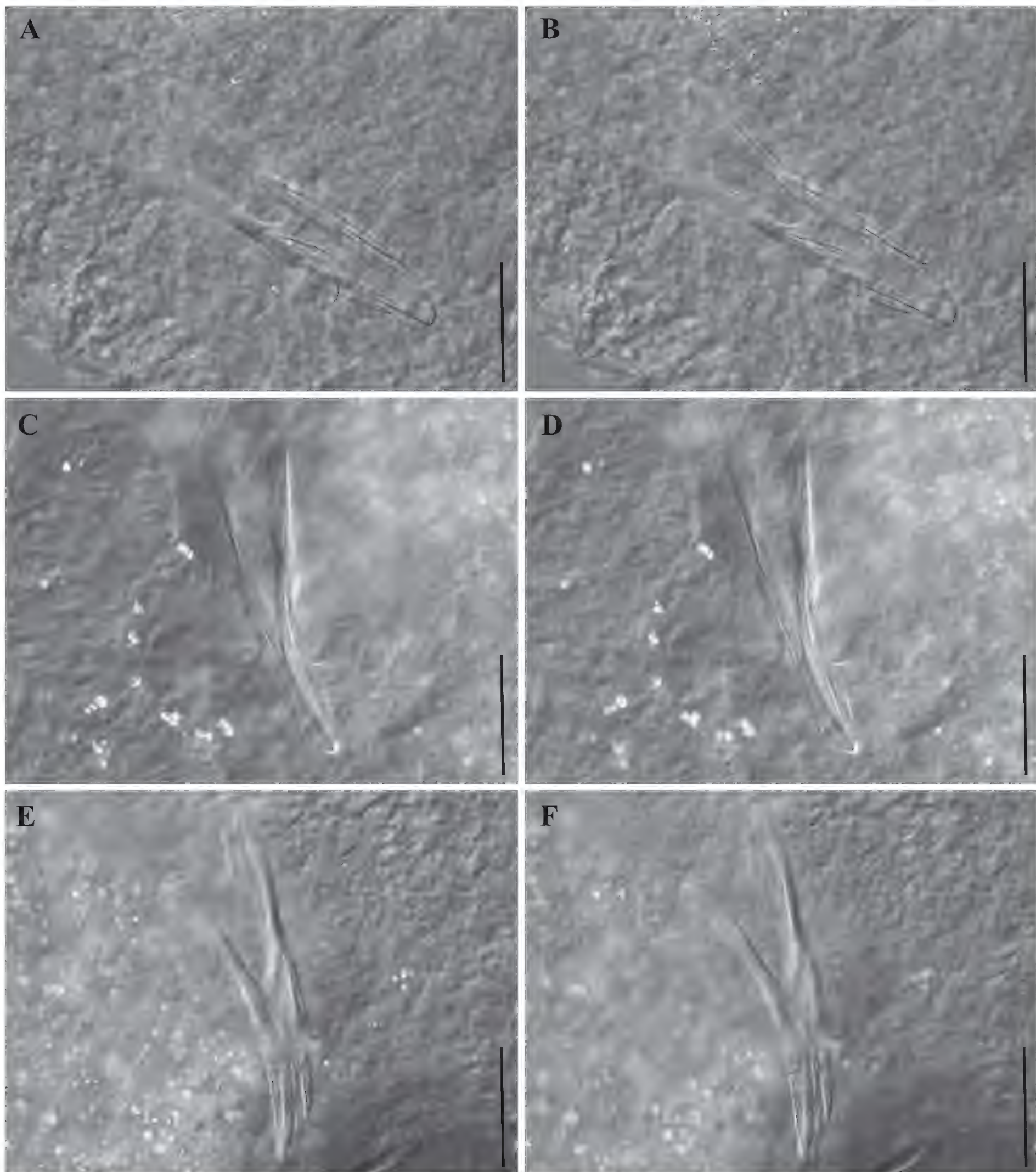


Figure 10. *Microdalyellia schmidtii*. A–F. Male stylet. Scale bars: 50 µm (A–F).

Nasonov (1919, 1926) provides insight into the close relationship among the previously mentioned species, particularly in Russian populations. Some specimens of *M. schmidtii* from Russia (Nasonov 1919: fig. 8) resemble *M. kupelwieseri* due to the presence of a few smaller spines alongside the main spine of one arm (see Meixner 1915; Luther 1955; Young 1970). However, Nasonov (1926) also recorded specimens of *M. schmidtii* with a single spine per arm, some displaying transitional forms between extreme morphologies of *M. schmidtii* and *M. armigera*. Nevertheless, populations of *M. kupelwieseri*

from Germany (Rixen 1961), the United Kingdom (Young 1970), and Spain (Fariás et al. 1995) show morphological convergence, with stylets measuring 100–110 µm and carrying one spine in one arm and three in the other.

The Hamburg population of *M. schmidtii* is notable for its significantly larger stylet (140 µm) compared to other recorded populations: 92 µm in the United Kingdom (Young 1970), 66 µm in Finland (Luther 1955), and 100 µm in Germany (Rixen 1961). Stylet sizes in *M. kupelwieseri* populations are also smaller (100–110 µm). The stylet in the new studied material I (ZMH V13848–V13856)

of *M. kupelwieseri* populations displays a triangular spine in the bridge linking the two branches of the stylet (Fig. 11B, D: sp), a structure lacking in both *M. schmidtii* and *M. armigera*. In addition, one of the spiny arms of *M. kupelwieseri* carries three spines, whereas the number is always one in *M. schmidtii* from Hamburg. We suspect, therefore, that the high similarity between the two species and the sympatric distribution of both in some localities, contributed with their misidentification. Indeed, the re-examination of two specimens identified as *M. kupelwieseri*

collected in Belgium and stored in the reference collection of Hasselt University (VII.4.20 and VII.4.24) allowed their reclassification as *M. schmidtii*. This action is made considering that their stylets lack a triangular spine in the bridge. A third specimen collected in Belgium, together the two previously mentioned, and sharing the same morphology, was included in the phylogenetic analysis by Van Steenkiste et al. (2013) and it clustered in our phylogeny with specimens of *M. schmidtii* collected in Hamburg (see section Molecular Phylogenetic Analyses; Fig. 16).

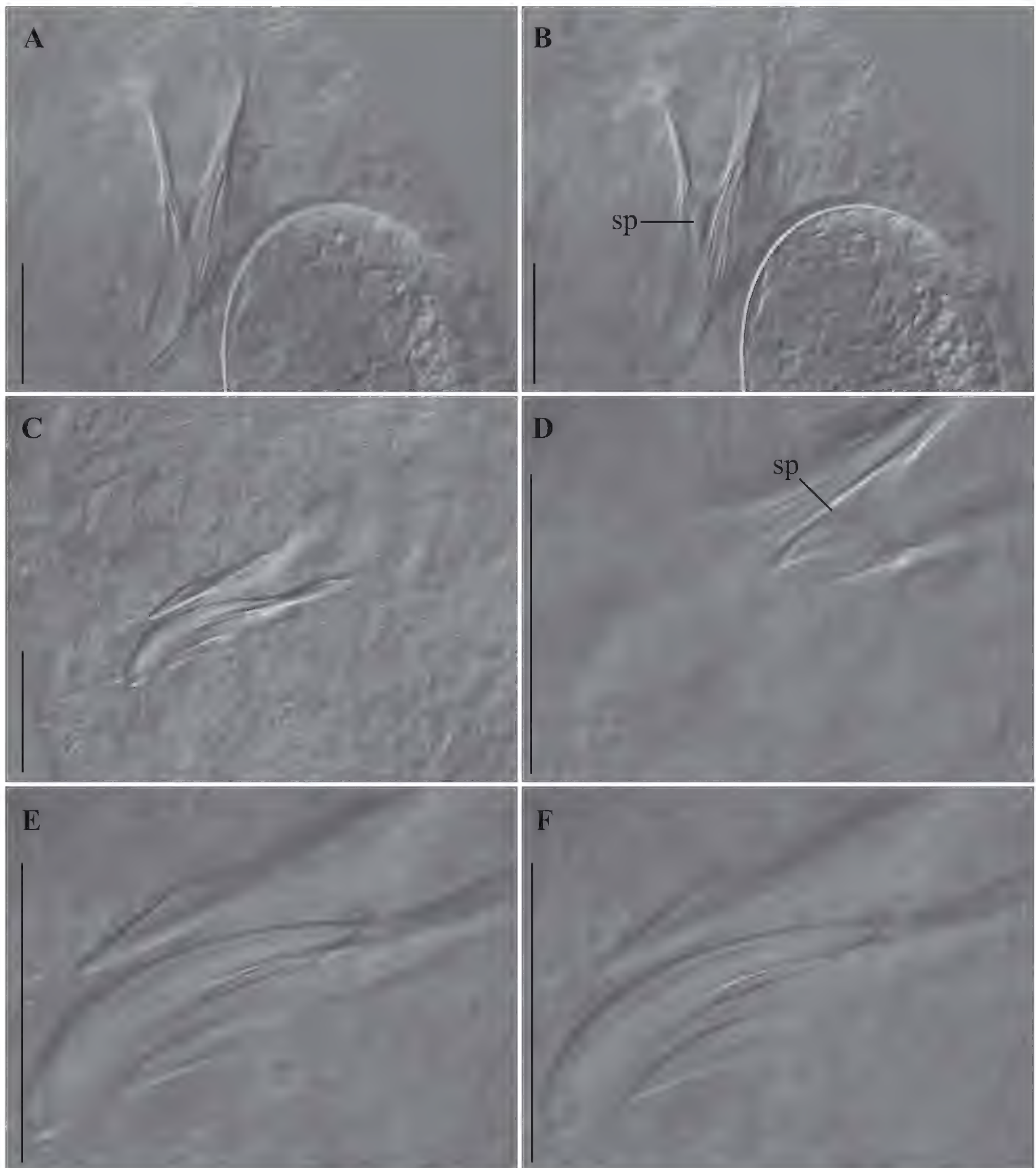


Figure 11. *Microdalyellia kupelwieseri*. **A–F.** Male stylet; **D.** Detail of the bridge's spine; **E–F.** Detail of the spiny branches. Scale bars: 50 μ m (**A–F**).

These findings support that the presence or not, of a triangular spine in the stylet's bridge is a diagnostic character for *M. kupelwieseri* and *M. schmidtii*, respectively.

Until now, the relationships among *M. armigera*, *M. kupelwieseri*, and *M. schmidtii*, and the validity of the latter two species, remained unclear. However, it is suggested that the group *M. kupelwieseri* – *schmidtii* can be distinguished from *M. armigera* by the reduced spine number in one arm, as well as the larger size of the single spines in *M. schmidtii* and the main spines of *M. kupelwieseri* compared to those in *M. armigera*. In this sense, our phylogenetic analysis contributed to clarify that the three species represent distinct lineages and support their validity. However, *M. armigera* could represent a complex of cryptic species (see section Molecular Phylogenetic Analyses; Fig. 16).

Typhloplanidae Graff, 1905 ***Krumbachia* Reisinger, 1924**

***Krumbachia hiemalis* Schwank, 1979**

Fig. 12

Known distribution. Until now, this species was only known from its type locality in Schlitz, Hessen, Germany (Schwank 1979).

Material. Sixteen specimens studied alive, five of them whole mounted (ZMH V13843–13847), nine preserved for future histological, and two used for molecular studies. Animals collected in Wittenberg, Rissen, on tree holes filled with water and litter, 20–50 cm over the ground level.

Description. Live animals 1.5–2 mm long, unpigmented and without eyes (Fig. 12A). Strong adhenal rhabdite tracts (Fig. 12B: ar) open in the anterior body end. Body oval-elongated, with the anterior margin broader than the posterior. The pharynx (Fig. 12A, C: ph) is located about midbody, 285 µm in diameter (all provided measures of this species are based on a single living specimen).

The testes (Fig. 12A: t) are located anterior and the ovary and atrial organs posterior to the pharynx. The male copulatory organ (Fig. 12A, C: mco) is 156 µm long and encompasses a seminal vesicle, a prostate vesicle, the sclerotised ejaculatory duct, and a bundle of accessory glands. The seminal vesicle (Fig. 12D, E: sv) receives independently both spermatid ducts and, then, opens into the prostate vesicle. The prostate vesicle (Fig. 12D, E: pv) contains a granular secretion, also observable throughout the ejaculatory duct. The ejaculatory duct (Fig. 12D, E: ed) is 102 µm long, with slightly sclerotised walls (not observable on the whole mounts). The distal half of the male copulatory bulb is occupied by a mass of accessory glands (Fig. 12D, E: gl), surrounding the ejaculatory duct.

The vitellaria (Fig. 12A: vi) extend at the body sides, between the brain and the posterior part of the body. The ovary (Fig. 12A, C: ov) is 152 µm long, with the oocytes increasing in diameter from proximal to distal. The female duct connects the ovary to the female atrium. A sem-

inal reservoir (Fig. 12A: sr) opens into the female duct (no sperm observed in this structure). The bursa (Fig. 12A, C–E: b) opens into the common general atrium in between the apertures of the female and male atria. Several vesicles were observed within the bursa but no sperm.

The morphology of the specimens collected in Hamburg closely aligns with the description outlined by Schwank (1979). For specimens from the type locality, the copulatory bulb length falls within the range of 120–180 µm. Additionally, Schwank (1979) noted the proximal oblique opening of the sclerotised duct in this species, a characteristic clearly observable in our recently collected specimens. This species stands out from all others within the genus *Krumbachia* due to the distinctive combination of a two-layered cuticularised ejaculatory duct, notable for its high mobility, and a bursa characterised by a remarkably thin wall.

Tricladida Lang, 1884

Continenticola Carranza, Littlewood, Clough, Ruiz-Trillo, Baguna & Riutort, 1998

Planarioidea Stimpson, 1857

Planariidae Stimpson, 1857

***Planaria* Müller, 1776**

***Planaria torva* (Müller, 1773) Müller, 1776**

Fig. 13

Known distribution. Species broadly distributed in West Europe (United Kingdom, Ireland, Belgium, Finland, Sweden, Denmark, Germany, Greece, France, and Italy) (Arndt 1926; Luther 1961; Ronneberger 1975; Ball and Reynoldson 1981; Müller and Faubel 1993; Martin and Brunke 2012), East Europe (Estonia, Latvia, Lithuania, Ukraine, Poland, and Czech Republic) (Luther 1961; Pinchuk 1979), and Russia (Grimm 1877; Luther 1961).

Material. Six specimens studied alive and preserved in absolute ethanol for future molecular analyses; one collected in Wandse river, submerged vegetation with organic matter, 0.1 m deep; one in Planten un Blomen park, submerged litter, 0.3 m deep; and four in Kirchwerder-Fünfhausen, submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep.

Remarks. Live adult specimens measuring 0.5–1.5 cm, dark pigmented, with a pair of anterior eyes (Fig. 13A–C: e). Squeezed specimens show the pharynx and atrial organs. The pharynx (Fig. 13C, D: ph) is located in the second body half and the mouth opens anterior to the male copulatory organ (Fig. 13A, C, D: mco). The seminal ducts form false seminal vesicles (Fig. 13A, C, D: fsv) beside the anterior part of the pharynx. The male copulatory organ receives medially, independently, both seminal ducts, which evacuate the sperm in a single proximal seminal vesicle. Distally, the bulb forms a muscular penial papilla. One adenodactyl (Fig. 13C, D: ad) opens into the common atrium, at the right side of the male bulb; it is oriented backwards. The adenodactyl is

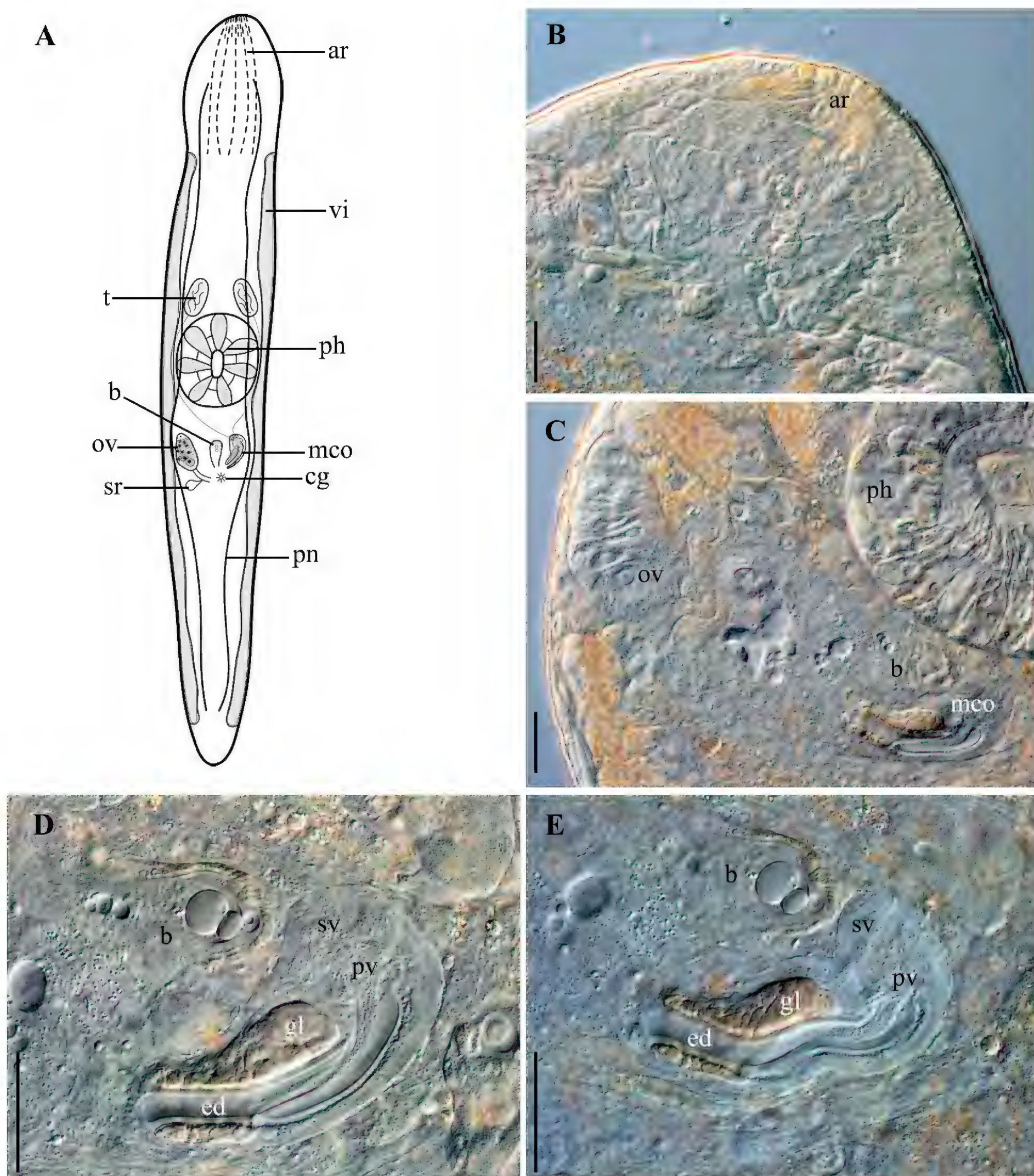


Figure 12. *Krumbachia hiemalis*. **A.** Habitus of live specimens; **B.** Anterior region; **C.** Posterior region; **D, E.** Atrial organs. Scale bars: 50 μ m (**B–E**).

distally bent and the central lumen makes it look hollow. The single observed structure of the female system was the bursa (Fig. 13D: b), located to the right side of the male organ.

Planaria torva is a species widely distributed throughout West Europe, and it is frequently mentioned in taxonomic literature on triclad in the region. However, accurate identification of this species can be challenging without a detailed examination of internal morphology. The taxonomic history of *P. torva* has

been contentious, with several studies mistakenly associating it with species of *Dugesia* (Ball et al. 1969). In light of these issues, we based the identification of our specimens on descriptions provided by Luther (1961) and Ball et al. (1969). The presence of an adenodactyl serves as a key distinguishing feature between our studied specimens of *P. torva* and species of *Dugesia* (Luther 1961). Furthermore, the overall structure of the atrial organs in our specimens, particularly the male bulb and the adenodactyl, unequivocally supports their

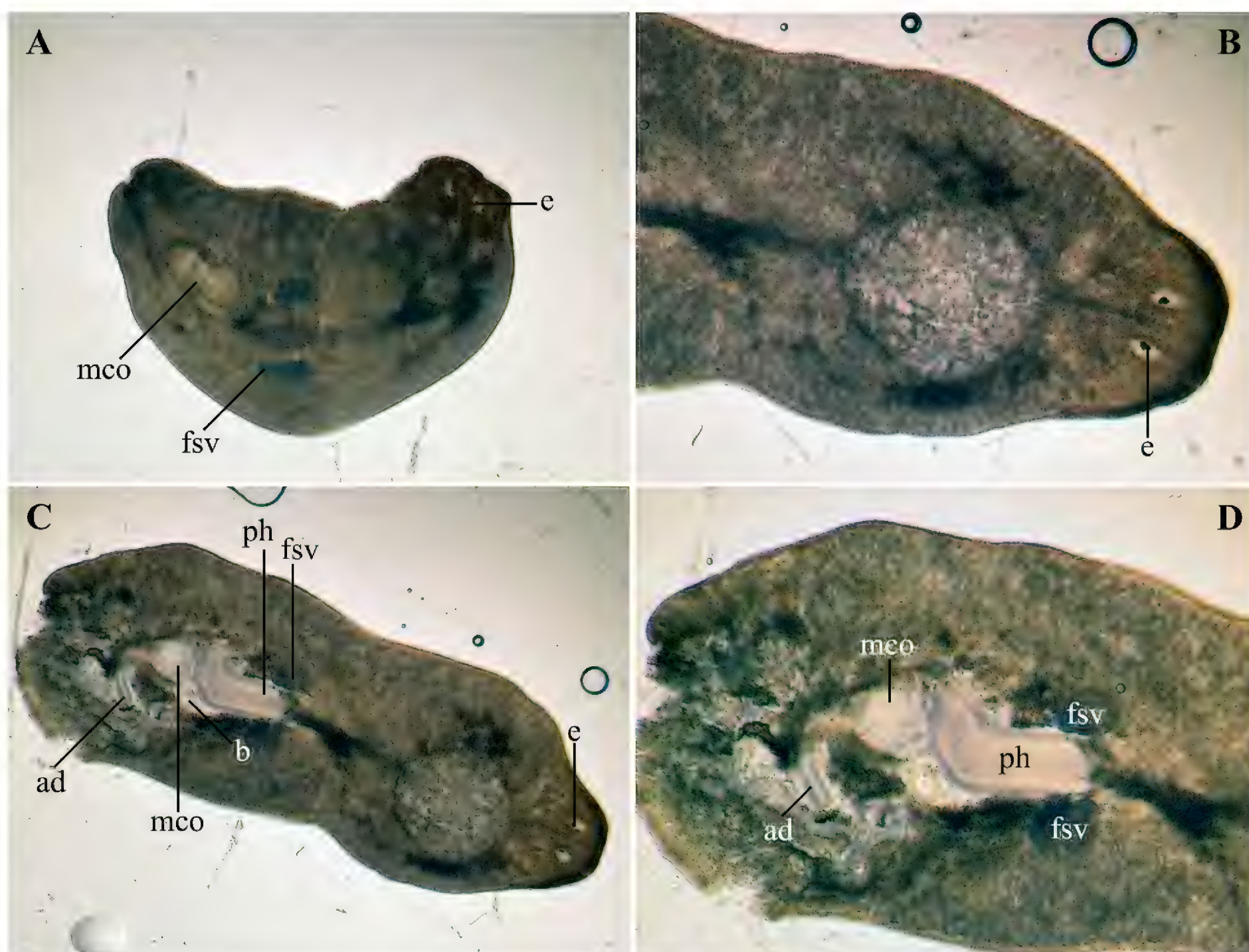


Figure 13. *Planaria torva*. **A.** Habitus of swimming specimen; **B–D.** Squeezed specimen.

classification within *P. torva*. For a more comprehensive comparison of this species with related congeners, refer to Ball et al. (1969).

Polycelis Ehrenberg, 1831

Polycelis tenuis Ijima, 1884

Fig. 14

Known distribution. Species recorded from The Netherlands (Young 1972), Finland, United Kingdom and Ireland (Luther 1961), Germany (Schwank 1981; Martin and Brunke 2012), Macedonia (Kenk 1978), Romania (Felićia 2018), and Russia (Luther 1961).

Material. Six specimens studied alive, preserved in absolute ethanol for future molecular analyses; collected in Kirchwerder-Fünfhausen, submerged vegetation and litter in an irrigation channel, 0.1–0.2 m deep.

Remarks. Mature specimens measuring 0.5–1.2 mm, dark coloured (Fig. 14A, B). Marginal eyes (Fig. 14C, D: e) distributed over the anterior third of the body. The pharynx (Fig. 14B, C) is located over the midbody and the mouth (Fig. 14C, E: m) opens anterior to the male copulatory organ. The male copulatory organ (Fig. 14B, E: mco) is 940–1100 µm long ($n = 1$; varying according to

the relaxing stage) and 620 µm at widest. The male organ is spiny over its distal 300–480 µm, and forms a penial papilla (Fig. 14E: pp). The spines (Fig. 14F) are 17–18 µm long ($n = 10$). Two adenodactyls (Fig. 14E: ad) are located posterior to the male bulb and open into the common atrium, oriented forward, and exhibiting a glandular lumen.

Three species of *Polycelis* have been documented in Germany, and they are widespread across Europe: *P. felina*, *P. nigra*, and *P. tenuis* (Volk 1903; Ronneberger 1975; Schwank 1981; Müller and Faubel 1993). Among these, only *P. nigra* has been reported in Hamburg (Volk 1903). Species within the genus *Polycelis* are primarily distinguished by the structure of the male bulb and adenodactyls. *Polycelis tenuis* shares with *P. felina* the presence of two adenodactyls, structures absent in *P. nigra*. However, *P. felina* is easily identifiable by the presence of two tentacles in its anterior body region. The penial papilla of *P. tenuis* is armed with spines along its distal half, whereas *P. nigra* exhibits two to three spine rows distally, and *P. felina* lacks any spines in this region (Hansen-Melander et al. 1954; Luther 1961; Harrath et al. 2012). Volk (1903) did not provide detailed morphological information about the specimens of *P. nigra* recorded in Hamburg. Given the necessity of studying the morphology of atrial organs for accurate identification of these triclads, this record requires confirmation.

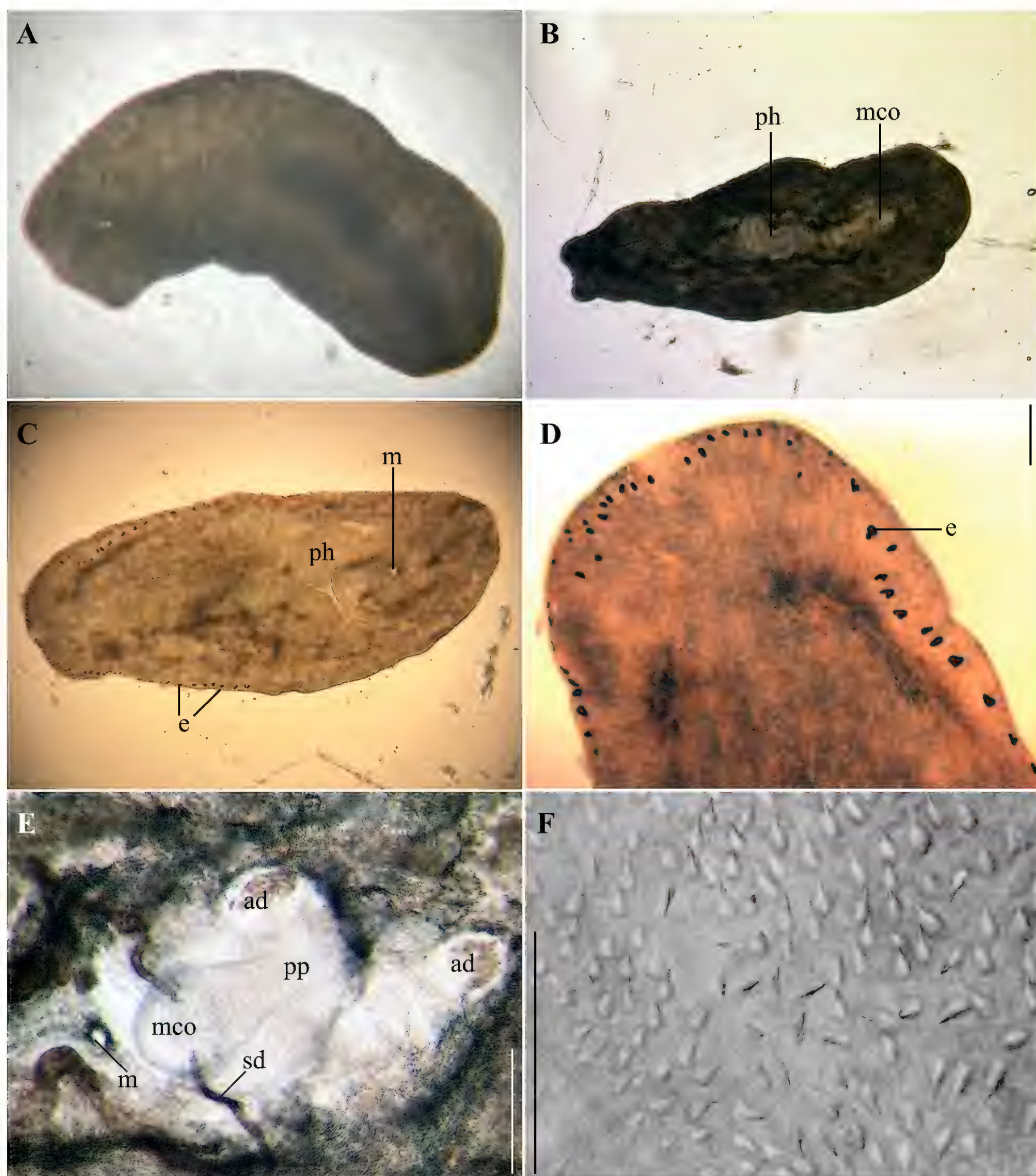


Figure 14. *Polycelis tenuis*. **A.** Habitus of swimming adult specimen; **B.** Squeezed adult specimen; **C, D.** Squeezed juvenile; **E.** Atrial organs; **F.** Spines of the penial papilla. Scale bars: 200 µm (**D**); 600 µm (**E**); 100 µm (**F**).

Molecular phylogenetic analyses

Catenulida

The dataset of Catenulida included sequences of 56 specimens, representing at least 14 species of *Stenostomum* and two outgroups. Eight of these specimens were sequenced for the present study. After removing ambiguously aligned positions, the 18S, 28S rDNA, and COI mDNA alignments were 1612, 1473 and 553 bp long,

respectively, resulting in a concatenated alignment of 3615 bp. Bayesian and ML topologies were congruent after collapsing of weakly supported clades. The resulting phylogeny of the analysis is shown in Fig. 15.

All species of *Stenostomum* cluster in a highly supported clade (pp = 1; SH-aLRT = 98.8; UFboot = 98). *Stenostomum arevaloi* resulted as the sister to all other species of the genus included in the analysis (pp = 0.99; SH-aLRT = 47.5; UFboot = 63). Species within this large clade cluster into two groups, one containing *Stenostomum handoelense*,

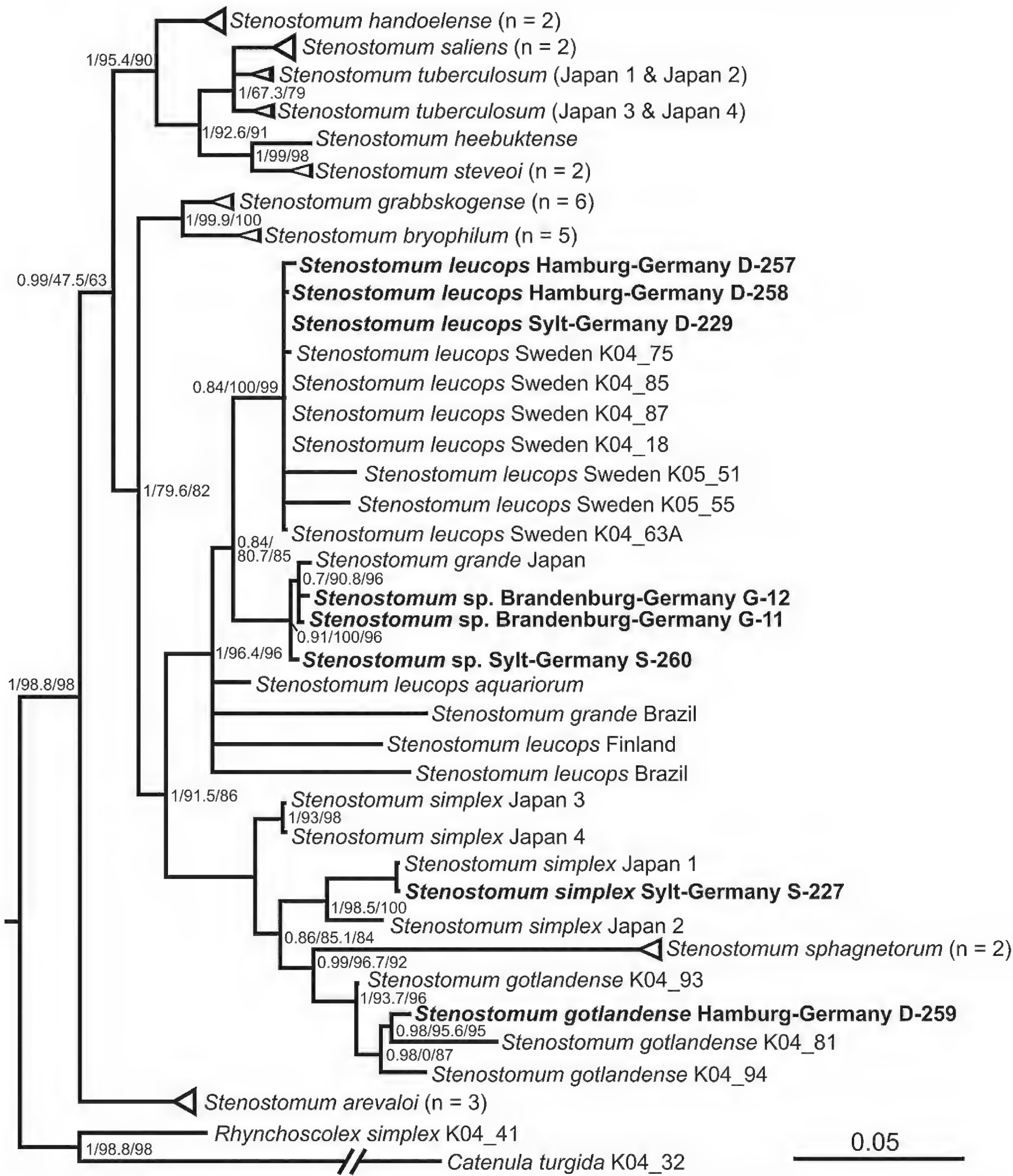


Figure 15. Majority-rule consensus tree from the Bayesian analysis of the concatenated 18S + 28S rDNA + COI mDNA dataset of *Stenostomum*, Catenulida. Branches with support values below the thresholds in the legend of the three analyses were collapsed. Support values are represented in the order of posterior probabilities / SH-aLRT / ultrafast bootstrap. Branches without symbols have pp = 1, SH-aLRT = 100, and UFboot = 100. Taxa from which new sequences were obtained for this study are highlighted in bold.

S. saliens, *S. tuberculosum*, *S. heebuktense*, and *S. steveoi* (pp = 1; SH-aLRT = 95.4; UFboot = 90); and the other (pp = 1; SH-aLRT = 79.6; UFboot = 82) including three subgroups: *S. grabbskogense* + *S. bryophilum* (pp = 1; SH-aLRT = 99.9; UFboot = 100), *S. leucops* + *S. grande* + *S. leucops aquariorum* (pp = 1; SH-aLRT = 96.4; UFboot = 96), and *S. simplex* + *S. sphagnetorum* + *S. gotlandense* (pp =

1; SH-aLRT = 100; UFboot = 100). The specimens of *S. tuberculosum* form two distinct clades within a polytomy with *S. saliens* (pp = 1; SH-aLRT = 67.3; UFboot = 79). Specimens of *S. leucops* form four lineages: one including specimens from Germany and Sweden (pp = 0.84; SH-aLRT = 100; UFboot = 99), and the others corresponding to single specimens from Finland (*S. leucops* and *S. leu-*

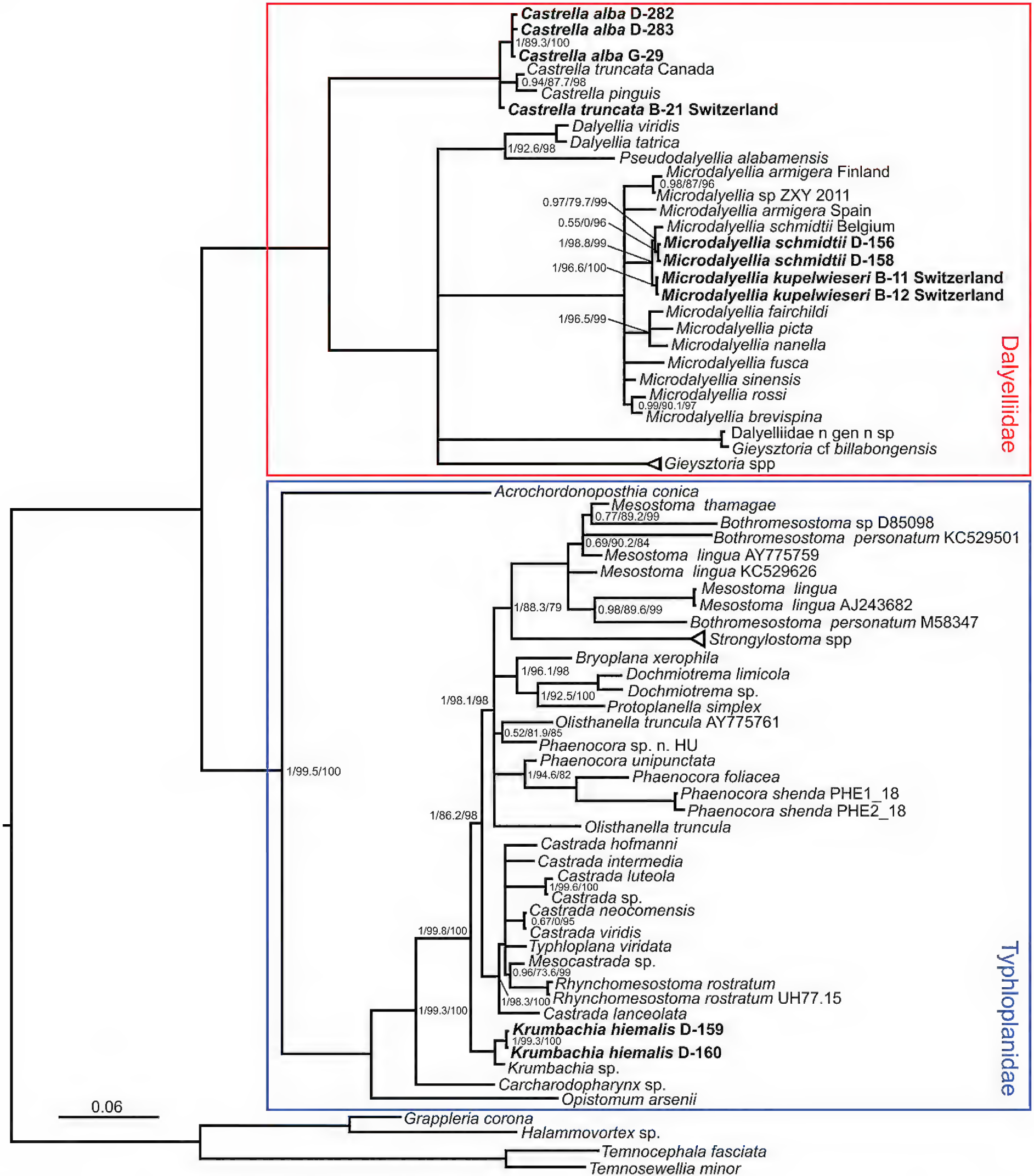


Figure 16. Majority-rule consensus tree from the Bayesian analysis of the concatenated 18S + 28S rDNA dataset of Rhabdocoela. Branches with support values below the thresholds in the legend of the three analyses were collapsed. Support values are represented in the order of posterior probabilities / SH-aLRT / ultrafast bootstrap. Branches without symbols have pp = 1, SH-aLRT = 100, and UFboot = 100. Taxa from which new sequences were obtained for this study are highlighted in bold.

cops aquariorum) and Brazil. One specimen of *S. grande* from Japan and three specimens of an unidentified species from Germany cluster together (pp = 0.91; SH-aLRT = 100; UFboot = 96) and this clade is sister to that including the specimens of *S. leucops* from Sweden and Germany (pp = 0.84; SH-aLRT = 80.7; UFboot = 85).

Stenostomum simplex forms two clades, one including specimens from Japan (Japan 3 and 4 in Fig. 15)

(pp = 1; SH-aLRT = 93; UFboot = 98) and the other including specimens from Japan (Japan 1 and 2) and Germany (pp = 1; SH-aLRT = 99.6; UFboot = 100). This last group of *S. simplex* is sister to *S. sphagnetorum* + *S. gotlandense* (pp = 0.99; SH-aLRT = 96.7; UFboot = 92). *Stenostomum gotlandense* includes specimens from Germany and Sweden (pp = 1; SH-aLRT = 93.7; UFboot = 96).

Rhabdocoela

The dataset of Rhabdocoela included sequences of 97 specimens, representing 84 species. Ten of these specimens were sequenced for the present study. After removing ambiguously aligned positions, the 18S and 28S rDNA alignments were 1793 and 1743 bp long, respectively (concatenated alignment of 3536 bp). Bayesian and ML topologies were congruent after collapsing of weakly supported clades. The resulting phylogeny of the analysis is shown in Fig. 16.

As previously shown by Van Steenkiste et al. (2013), both ‘Typhloplanidae’ (pp = 1; SH-aLRT = 99.5; UFboot = 100) and Dalyelliidae (pp = 1; SH-aLRT = 100; UFboot = 100) form two highly supported clades (Fig. 16). Within ‘Typhloplanidae’, our newly generated sequences of *Krumbachia hiemalis* cluster together with *Krumbachia* sp. and provide the first molecular support for the monophyly of the genus (pp = 1; SH-aLRT = 99.8; UFboot = 100). Otherwise, the genera *Mesostoma*, *Bothromesostoma*, *Phaenocora*, *Olisthanella*, and *Castrada* were recovered as paraphyletic. Three sequenced specimens of *Bothromesostoma*, two of them identified as *B. personatum*, are deeply embedded within *Mesostoma*. Specimens of *Mesostoma lingua* do not form a monophyletic clade.

Species of *Castrella* cluster in a fully supported clade (pp = 1; SH-aLRT = 100; UFboot = 100) which is the sister taxon to all other dalyelliids (pp = 1; SH-aLRT = 100; UFboot = 100). Three specimens of *C. alba* cluster together (pp = 1; SH-aLRT = 89.3; UFboot = 100) and form an unresolved group with *C. truncata* from Switzerland and *C. pinguis* + *C. truncata* from Canada (pp = 94; SH-aLRT = 87.7; UFboot = 98). Therefore, this analysis revealed the existence of cryptic diversity within *C. truncata*, containing, at least, an European and a North American lineages.

The second clade within Dalyelliidae forms a polytomy including the groups of most species of *Gieysztoria* (pp = 1; SH-aLRT = 100; UFboot = 100), species of *Dalyellia* + *Pseudodalyellia alabamensis* (pp = 1; SH-aLRT = 92.6; UFboot = 98), Dalyelliidae n. gen. n. sp. + *Gieysztoria* cf. *billabongensis* (pp = 1; SH-aLRT = 100; UFboot = 100), and species of *Microdalyellia* (pp = 1; SH-aLRT = 100; UFboot = 100). The interrelationships of two analysed specimens of *M. armigera* are not fully resolved. Otherwise, specimens of *M. kupelwieseri* and *M. schmidtii* cluster together (pp = 1; SH-aLRT = 98.8; UFboot = 99). One specimen of *M. schmidtii*, collected in Belgium, clusters with two specimens of the same species collected in Hamburg (pp = 0.97; SH-aLRT = 79.7; UFboot = 99) and together are sister to a clade containing two specimens of *M. kupelwieseri* collected in Basel, Switzerland (pp = 1; SH-aLRT = 96.6; UFboot = 100).

Discussion

There is no doubt that Germany boasts one of the most thoroughly researched and understood diversities of turbellarians worldwide. However, the distributional knowledge

of this diversity varies significantly among different regions within Germany. Consequently, it is not uncommon to encounter new species records in areas with relatively limited sampling efforts. Undoubtedly, the sparse studies conducted in Hamburg and the limited number of species recorded highlight this region as poorly explored in terms of turbellarian diversity within Germany. It is necessary to emphasize the rich diversity of freshwater turbellarians found in urban and suburban environments of Hamburg, evidencing their importance for conservation. Particularly, the shallow irrigation ditch sampled in Kirchwerder-Fünfhausen hosted the highest found richness with eight species. Therefore, we suspect that a much higher diversity can be detected in a broader collecting campaign.

Our discoveries have led to the record of two already known catenulids for Germany, *Stenostomum gotlandense* and *S. simplex*. A third species, provisionally identified as *Stenostomum* sp., is related to Japanese specimens of *S. grande* (see Fig. 15), a species not recorded from Germany. *Stenostomum* represents a genus of turbellarians that remains poorly studied and encompasses several taxa in need of revision, likely containing cryptic species. Therefore, the information we have provided on German species of *Stenostomum* holds significance for future investigations into these species.

For instance, our study supports previous findings (i.e. Rosa et al. 2015) suggesting that *S. leucops* and *S. grande* represent two complexes of cryptic species, a proposition supported by the morphological variability observed in our examined specimens compared to those documented in the literature. As such, *S. grande* is represented in our study by two lineages, one from Brazil and the other including specimens from Germany and Japan. Within the four lineages representing specimens of *S. leucops*, one is morphologically remarkable, *S. leucops aquariorum*. This subspecies was described by Luther (1960) from Helsinki, Finland, and is diagnosable by a reduced number of spherules (6–10) contained in the refractile bodies, whereas there are more than 20 in the nominal species. Consequently, given the importance of the refractile bodies’ morphology to differentiate catenulid species and the evidences of our phylogenetic analysis, we propose to recognise *S. leucops aquariorum* as a distinct and valid species of *Stenostomum*, now *S. aquariorum* stat. nov. (for the description of the species see Luther 1960).

The cryptic diversity observed within the *S. leucops* – *S. grande* group appears to also apply to *S. simplex*. Specimens of *S. simplex* from Japan do not form a monophyletic clade, with two of them clustering alongside a specimen from Germany. As a result, our identification of the German specimen as *S. simplex* is provisional and requires further confirmation through more extensive morphological and phylogenetic analyses. The type localities of the three species discussed here—*S. leucops*, *S. grande*, and *S. simplex*—are all located in the United States. Therefore, studying specimens from their type localities is crucial to accurately delineate species boundaries. In contrast, *S. tuberculosum* shows a different pattern,

with two distinct clusters emerging from our analysis. This divergence may be influenced, and potentially artificially generated, by the fact that two specimens are represented by partial 18S rDNA sequences (Japan 3 and 4), while the other two are represented by partial COI mDNA sequences (Japan 1 and 2).

Similarly, as occurs with species of *Stenostomum*, *Pro-rhynchus stagnalis* represents another case of cryptic diversity (Tyler et al. 2018; Reyes et al. 2021), evidenced in the size variability of the stylet in the different studied populations. Furthermore, the German populations of *Gyratrix hermaphroditus* warrant scrutiny in light of the findings by Tessens et al. (2021), who revealed this species as the largest known case of cryptic diversity among microturbellarians. Specifically, a re-evaluation of specimens from the type locality (Berlin) is highly recommended before proceeding with the description of additional species.

Our phylogenetic analysis suggests that *Bothromesostoma* should be synonymized with *Mesostoma*. Braun (1885) distinguished these two genera based on the morphology of the testes and the presence of a ventral, epidermal follicle (“Hautfollikel”): species of *Mesostoma* were characterised by two compact testes and the absence of a follicle, whereas species of *Bothromesostoma* had follicular testes and a ventral, epidermal follicle (“glandular sac” as termed by Marcus 1946, 1960). However, these traits appear to have evolved or been lost multiple times within the group. For instance, *M. macroprostatum* Hyman, 1939 also exhibits a glandular sac anterior to the pharynx. Marcus (1955) additionally noted that species of *Bothromesostoma* possess a spermatic duct between the bursal canal and the female genital canal, a structure absent in other mesostomids, which are classified under *Mesostoma*. Nevertheless, he also recognised significant variability in other morphological features within *Mesostoma*. Thus, the presence of a spermatic duct and glandular sac may simply reflect interspecific variation within *Mesostoma*, rather than defining features of distinct genera. Our phylogenetic analysis also revealed the presence of cryptic diversity within this group, identifying two distinct lineages of *B. personatum* and three of *M. lingua*. However, this observed cryptic diversity may be attributed to misidentification, given the highly homogeneous external morphology of these species. A similar situation, potentially involving either cryptic diversity or species misidentification, was noted in the two lineages of *Olisthanella truncula* and *Phaenocora* that were recovered in our analysis.

Hamburg specimens of *Microdalyellia schmidtii* stand out due to their stylet morphology, notably larger than that found in other populations of the species and its closely related counterpart *M. kupelwieseri*. Specimens of *M. schmidtii* were discovered alongside *Krumbachia hiemalis* in phytotelmata, which are tree holes containing water, a habitat that has received relatively little exploration in terms of meiofauna diversity (see Majdi et al. 2024). Since turbellarians inhabit a wide range of aquatic and limnoterrestrial ecosystems, it is crucial to include

phytotelmata in studies, as these habitats likely host a diverse array of species. Furthermore, our examination of specimens of *K. hiemalis* marks only the second recorded instance of the species since its initial description by Schwank (1979).

Our developed phylogenetic analysis illuminated the previously questioned validity of *M. schmidtii* and *M. kupelwieseri*, two species suggested as forms of *M. armigera*. Clearly, *M. armigera* forms distinct lineages with respect to the other two species, and, at the same time, seems to contain more than one species due to the different Finnish and Spanish lineages. Therefore, considering the broad distribution of *M. armigera* and the high morphological variability recorded for its populations (regarding the stylet size and spines number and size), it is indispensable to conduct a broad and integrative taxonomical study to clarify its diversity. On the other hand, the well-supported clades of *M. schmidtii* and *M. kupelwieseri* are morphologically diagnosable due to the spine present in the stylet’s bridge of *M. kupelwieseri*, a structure missing in *M. schmidtii*.

Freshwater triclads have been extensively studied across Europe but recent studies revealed several undescribed species, particularly of *Dugesia*, and a complex biogeographical and evolutionary history (Benítez-Álvarez et al. 2023; Dols-Serrate et al. 2023). However, the two triclads we collected in Hamburg have a history of misidentification due to the lack of studies on their internal morphology. Identifying these species without examining their copulatory organs is nearly impossible and has proven to be detrimental, leading to numerous unnecessary taxonomic challenges. Therefore, confirming the distribution of *Polycelis nigra* in Hamburg requires examining fresh material, as Volk (1903) did not provide morphological evidence for its classification. In fact, the species we found in Hamburg is *P. tenuis*, easily distinguishable by the presence of two adenodactyls and the spiny armature of the male copulatory organ.

In summary, our study sheds light on the complex and understudied diversity of turbellarians in Germany, particularly in Hamburg, where limited sampling has resulted in sparse species records. Our findings highlight the cryptic diversity within several genera, including *Stenostomum*, *Mesostoma*, and *Microdalyellia*, as well as the need for further research to clarify taxonomic boundaries, particularly in species with highly homogeneous morphologies. The morphological variability observed within *Stenostomum leucops*, *S. grande*, and *S. simplex* supports the existence of cryptic species, reinforcing the importance of comprehensive phylogenetic and morphological analyses, including studies of specimens from type localities. Additionally, our identification of *Microdalyellia schmidtii* and *Krumbachia hiemalis* in Hamburg’s unique phytotelmata habitats emphasizes the importance of exploring diverse aquatic environments to better understand the true extent of turbellarian diversity. Lastly, the need for accurate species identification through detailed morphological examination, as demonstrated in the case of *Polycelis nigra* and *P. tenuis*, remains critical for

avoiding taxonomic misinterpretation and advancing the knowledge of freshwater turbellarian biodiversity in Germany and beyond.

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